ANALYSIS OF SELECTED NATURAL COMPOUNDS AND THEIR DEGRADATION PRODUCTS IN PULP AND PAPER MILL EFFLUENT: EXPLORATION OF POSSIBLE ENDOCRINE DISRUPTORS

By

BRIAN QUINN

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2004

ACKNOWLEDGMENTS

There are many people who have had a hand in making my research projects successful, and I am grateful to each of them for their efforts. I would like to start by thanking my committee (Drs. Joe Delfino, Tim Gross, Paul Chadik, and Dave Powell) who have taken time out of their busy schedules to guide my research and scholastic training. I have benefited from each member's knowledge and experience. I would especially like to thank my supervisory committee chair, Dr. Delfino, who helped guide my experiments, and taught me a little diplomacy; and my cochair, Dr. Gross, who also played a large role in experimental design, and kept my salary coming well after my funding had ended.

I would like to thank Dr. Matt Booth for his long hours analyzing my samples, helping with data interpretation, and his dedication to quality work. I thank Dr. Dave Mazyck for allowing me to conduct radiological experiments under his auspices. I thank Dr. Margaret James for her excellent advice, and references on liver dysfunction in vertebrates. I extend my gratitude to Drs. Jodie Johnson and Angela Lindner for their help with the doomed LC/MS system.

I thank the field crew at USGS for their countless hours of work, including Carla Wieser who helped me with all laboratory issues; Jessica Noggle, a fellow graduate student and compatriot on the biological side of these studies; and Shane Ruessler, a great friend who has worked long and hard to keep me sane.

I would like to thank the Georgia-Pacific Corporation for their monetary support during the early part of this project, and especially Stewart Holm and Myra Carpenter for their assistance at the mill in Palatka. I extend my thanks to Buckeye Cellulose for monetary support, and especially Chet Thompson and Greg Wynn for their help in the field and data collection.

I would like to thank all of the students with whom I worked and played. I learned so much from each encounter with my peers, both culturally and academically. I thank all of the scientists whose work I used to build these studies. All of their hard work and dedication gave me the knowledge to analyze and interpret my data. I would like to thank one scientist in particular, Dr. Carl Miles, who convinced me to quit my job, move to Gainesville, and go to graduate school, while also convincing Dr. Delfino to take on a new graduate student with average undergraduate grades. Carl died of cancer just before I began this project, and I can only hope that this body of work would meet his standards.

Finally, I would like to thank my family. My folks always facilitated reading and learning in their home. They taught me that one could learn by listening to many different people, from the guy sweeping the floor to the brightest college professor. I thank my wife, Nikki, for enduring the last 4 years with me, every step of the way; and I thank my dogs Cay and Al (who has survived a liver disease against all odds) for both being there with wagging tails whenever I arrived.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

ANALYSIS OF SELECTED NATURAL COMPOUNDS AND THEIR DEGRADATION PRODUCTS IN PULP AND PAPER MILL EFFLUENT: EXPLORATION OF POSSIBLE ENDOCRINE DISRUPTORS

By

Brian Quinn

August 2004

Chair: Joseph J. Delfino
Cochair: Timothy S. Gross
Major Department: Environmental Engineering Sciences

One objective of this study was to determine the favorable effects of process changes on largemouth bass at Georgia-Pacific's Palatka mill operation, a bleached/unbleached Kraft pulp and paper mill, using multiple chemical markers. These process changes, which included fixing leaks into the brown stock washer sewers, installing a new bleach plant using primarily chlorine dioxide, new condenser strips, and increased aeration in retention ponds, have been implemented to improve the quality of the effluent discharged to Rice Creek and, ultimately, the St. John's River. Three selected resin acids (including isopimaric, dehydroabietic, and pimaric acids); and four phytosterols (including stigmasterol, stigmastanol, campesterol, and β-sitosterol) were used as chemical markers to monitor the effects of process changes in the effluent, and in the bile of largemouth bass (*Micropterus salmoides*) during a 56-day exposure study. Results show that process changes decreased the concentrations of resin acids and

phytosterols in the effluent by nearly 80%. After process changes, largemouth bass exposed to the highest effluent concentration (80%) exhibited a 35-80% decrease in resin acid concentrations in bile, while phytosterol concentrations in bile decreased over 80% for all of the selected compounds.

Another objective was to assess the degradation of the phytosterol β-sitosterol using effluent-impacted water samples and upstream non-impacted reference samples. Degradation studies under aerobic and anaerobic conditions demonstrated that aerobic microbial metabolism was the dominant mechanism for compound breakdown. The half-life range for β-sitosterol was 22-28 days under aerobic conditions, and the degradation reaction rate followed first-order kinetics.

CHAPTER 1
LITERATURE REVIEW AND OBJECTIVES

Introduction

Paper and paper products are important commodities in our society. The US Environmental Protection Agency [US EPA, 1995] determined that 555 pulp and paper mills were operating in the US in 1992. In 1991, the world consumption of paper and paper products was 243 million tons, and the projected paper usage in 2010 is expected to be 440 million tons [Food and Agriculture Organization of the United Nations, 1994]. Unfortunately, pulp and paper production releases many compounds that pollute the waters receiving mill effluents [Richardson et al. 1983 and Suntio et al. 1988]. Identification, quantification, and environmental assessment of these pollutants are important steps in determining the potential environmental impacts of the pulp and paper industry.

Paper Production

The production of pulp and paper involves many varied and complex processes. These were summarized by the US EPA [1995] and are the basis of the following synopsis. After trees are felled and transported to pulp mills, they are debarked and chipped. After chipping, the wood fiber is screened, and the larger fibers are retained and recut to make a product of relatively uniform size called furnish. Furnish can then be pulped in a variety of ways. The most common type of pulping in the US is chemical pulping that includes the kraft and sulfite processes. Chemical pulping normally produces long and strong fibers that are used for finer papers and paper products.

Semichemical, mechanical, and secondary fiber pulping are different methods, but these produce shorter, weaker fibers that are used in products like newsprint paper, linerboard, and inexpensive paper towels. Georgia Pacific's Palatka Mill Operation (PMO), located near Palatka, Florida on the St. John's River system, is a kraft bleaching mill, so this process is discussed in detail below.

Kraft pulping starts with the addition of a mixture of Na_2S and NaOH (white liquor) to the furnish in the digester. Once the furnish is dissolved in the white liquor, it becomes a mixture of fibers called brown stock, (the desired product) and weak black liquor, which contains lignins and the initial white liquor components. The fiber is processed into pulp using screens and other physical methods; is cleaned in a brown stock washing area; and can then be bleached, if desired.

One of the most important aspects of the kraft process is that it regenerates pulping chemicals and energy. In this process, weak black liquor is added to an evaporator, to concentrate the mixture and to make strong black liquor. This strong black liquor is burned in a recovery boiler, creating energy for the mill; and results in a mixture called smelt. The smelt is then recausticized to convert Na_2CO_3 to NaOH, which is accomplished by mixing weak black liquor with the smelt, to form something called green liquor. The green liquor is mixed with CaO to produce the desired white liquor, and a precipitate called dregs (which consists largely of $CaCO_3$). The white liquor is reused in the pulping process, and the dregs are burned in a lime kiln to regenerate CaO.

Pulp is bleached to improve the brightness of paper products. Commonly, pulp is bleached first in an acidic environment; and then under basic conditions, it is washed between each bleaching stage. These processes are repeated using various bleaching

agents to attain the brightness desired by the manufacturer. The pH is varied during bleaching to remove acid-neutral and base-extractable compounds. Many bleaching agents have been used in mills including NaOH, elemental chlorine, chlorine dioxide, hypochlorous acid, sodium hypochlorite, calcium hypochlorite, oxygen, hydrogen peroxide, sulfur dioxide, sulfuric acid, and ozone. The bleaching sequence for the PMO bleach line before 2002 was $C_{90}d_{10}$EopHDp, where Cd represents a mixture of chlorine (C) and chlorine dioxide (d) in proportions designated by subscripts; Eop is extraction with alkali (E) and the addition of elemental oxygen (o) and hydrogen peroxide (p); H stands for hypochlorite; and Dp is chlorine dioxide with added hydrogen peroxide. This sequence now excludes elemental chlorine because the US EPA has prepared rules, called Cluster Rules [USEPA 1998], designed to reduce the production and release of chlorinated organic compounds into the environment. According to the Cluster Rules, the use of elemental chlorine in pulp bleaching ended in 2001. Chlorine dioxide was the replacement-bleaching agent because it is a strong oxidizing agent that forms chlorinated compounds at a reduced rate compared to elemental chlorine. After 2001, a common bleaching sequence used by paper mills is DEopD [Deardorff et al. 1998]. After the bleaching processes, the pulp can go through stock preparation, which includes pulp blending, dispersion in water, beating and refining, and addition of wet additives. Pulp goes through beating and refining to add density and strength; while wet additives like resins, waxes, clays, dyes, and inorganic salts are added to create the desired paper product.

Resin acids and other wood extractives are usually released into sewers in different places in the mill. Some liquid waste containing resin acids from brown stock

washers, bleach plants, and recovery boilers is released into the effluent. Biological treatment varies widely among pulp and paper mills, which affects the quality, and resin acid concentrations of their effluents.

Resin Acid Analysis

Resin acids were chosen as chemical markers (compounds used to measure exposure either qualitatively or quantitatively) to monitor fish exposure in these studies due to their abundance in the PMO effluent, and the available methodology found in the literature. These compounds are diterpenic acids that are produced naturally in vascular plants. Resin acids are placed in two groups: abietane acids like abietic acid, which contain conjugated double bonds; and pimarane acids like isopimaric acid, which do not contain conjugated double bonds. Since pulp and paper mills extract these compounds during the pulping process, some of these wood extractives are released into the environment via effluent streams [Peterman et al. 1980]. The process areas in pulp mills that are most likely to release wood extractives into the effluent are brown stock washing and pulp washing between bleaching cycles.

Resin acids have been useful chemical exposure markers in water, sediment and bile; and they have been measured in other matrices as well. Lee et al. [1997] found both abietic and dehydroabietic acids in traditional Chinese medications using a liquid chromatograph (LC) with both ultraviolet (UV) and fluorescence detectors. Up to 70 ppm of dehydroabietic acid was found in some medications. Weser et al. [1998], using a gas chromatograph/mass spectrometer (GC/MS), quantified dehydroabietic acid and similar compounds in embalming materials found in an Egyptian Pharaoh's tomb.

Many scientific studies involving paper mills use resin acids as target analytes in water, sediment, and fish bile. Rogers [1973] introduced XAD-2 ion-exchange resin as a medium for effluent extractions before analysis by GC/MS. He used the ion-exchange resin in conjunction with Sephadex to fractionate pulp and paper mill effluents for toxicity studies. In the past, because of the lack of commercial availability of resin acids, this method was used to isolate and purify these compounds in paper mill effluent. Voss and Rapsomatiotis [1985] determined the optimum pH for extraction of resin acids from mill effluent. They concluded that pH 9 yielded the best extraction efficiency; and also that at pH 9, labile resin acids reactive in acidic conditions (e.g., levopimaric, palustric, and neoabietic acids) could not form other resin acids like abietic and dehydroabietic acids. In addition, extraction of effluent at this pH produced less emulsion, making the procedure shorter and cleaner.

Another view of resin acid extraction was offered by the National Council for Air and Stream Improvement (NCASI). NCASI [1997] outlined a method that called for extracting effluents first at pH 4 and then at pH 2. The pH 4 extraction was added to an earlier NCASI method [NCASI 1986] to account for the resin acid degradation at lower pH values discussed earlier. This method was used in this study to extract and quantify resin acids. Koistinen et al. [1998] compared dichloromethane liquid-liquid extraction to semi-permeable membrane devices (SPMDs) which are clear polymer bags made of material similar to dialysis tubing that facilitate extraction and concentration of compounds that come in contact with it. Their results showed that liquid-liquid extraction and SPMD were very similar in the types of compounds isolated from the effluent matrices, but the SPMDs extracted a larger spectrum of compounds. Semi-

permeable membrane devices could be an effective tool in analyzing paper mill effluents, but they have limitations such as being easily overloaded in matrices containing high concentrations of organics; and inefficient extraction of substances bound to suspended solids such as cellulose fibers.

Richardson et al. [1983] first analyzed dehydroabietic acid by liquid chromatography (LC) using a fluorescence detector. They found that the limit of detection was 1 ng as compared to 7 ng using a UV detector. Suckling et al. [1990] methylated resin and fatty acids, and analyzed them using a liquid chromatograph connected to an evaporative light scattering detector (ELSD). An ELSD does not require that a compound contain chromophores, so compounds traditionally not seen using LC/UV analysis could be quantified, although an internal standard must be used because the ELSD is not linear for all compounds. A study conducted by Richardson et al. [1992] utilized C_{18} solid-phase cartridges for extraction of resin acids from effluent and formed coumarin derivatives using LC with post-column alkaline hydrolysis. Two different coumarin derivatives were investigated: one structurally designed for UV detection, and the other for fluorescence detection. Detection limits for UV and fluorescence were 20 μg/mL and 1 μg/mL, respectively. Researchers in one study injected paper mill effluent directly into a LC/UV instrument and found low responses for resin acids as compared to duplicate samples analyzed by a reference method [Chow and Sheppard 1996]. They found that resin acids adhere to suspended solids (such as paper fibers) at neutral and acidic pH values. Therefore, they added NaOH until the mill effluent sample reached pH 10, and then directly injected the mixture into the LC. Results were similar to those for samples analyzed by their reference method [Chow and Sheppard 1996].

Dethlefs and Stan [1996] used C_{18} and polystyrene divinylbenzene solid-phase cartridges to extract resin acids from effluents, and then derivatized the sample extracts to form pentafluorobenzyl esters for GC/MS analysis. They found that the method worked well for all resin acids except levopimaric acid, which isomerized into dehydroabietic acid during the solid-phase extraction step in the procedure. Arrabal and Cortijo [1994] extracted the heartwood of a Spanish pine tree using a Soxhlet extraction method, and removed the triglycerides by saponifying the sample extracts with ethanolic potassium hydroxide. Their results, using GC/MS, showed that abietic and dehydroabietic acids were the most abundant of the resin acids present in the wood extract.

Since resin acids are more likely to partition into sediments, extraction methodology for this matrix has been worked out using several different techniques. Lee and Peart [1992] used supercritical fluid extraction with methanol and formic acid as the extraction solvent mixture, to analyze sediments beneath waters receiving pulp and paper mill effluent. Sediment sample extracts were derivatized to form pentafluorobenzene esters, and analyzed by GC equipped with an electron capture detector (ECD). Method recoveries were good (88-102%) for all resin acids analyzed, except neoabietic acid and palustric acid. These both degraded into abietic acid due to the formic acid present in the extraction solvent mixture. Tavendale et al. [1995] outlined an extensive sediment extraction procedure designed to include chlorophenolic constituents, resin acids, and base-neutral resin-sourced cyclic hydrocarbons. The method uses Soxhlet extraction in combination with fractionation by gel permeation chromatography and different liquid-liquid extractions. Matrix recoveries of standards were 71-104% for many analytes of interest. Only two groups of analytes, vanillins and catechols, exhibited very poor

extractability from sediments using this method, showing method spike recoveries ≤ 21%. Judd et al. [1995] reported that most of chlorophenols and resin acids were found in surficial sediments (depth ≤ 3 cm) as compared to deeper sediments. Wood extractive compounds were also found in sediments that were not impacted by paper mills. Retene, tetrahydroretene, and dehydroabietin (all degradation products of dehydroabietic acid [Tavendale et al. 1997a]) were found in at least 43 of 310 aquatic sediment samples collected throughout Florida [Garcia et al. 1993].

Analyzing resin acids in bile is an important way to quantify exposure of fish to pulp and paper mill effluent. Fish liver analysis has been a more traditional approach for investigating exposure and bioconcentration of xenobiotic compounds; but fat-based compounds (such as triglycerides) present in the liver make isolation and quantitation of less polar target analytes a more difficult task. Bile contains little or no fat, and can easily be extracted with a minimum amount of emulsion being formed. Dehydroabietic acid was found in great abundance in blood plasma and liver tissue of rainbow trout, in an exposure study conducted by Oikari et al. [1982a]. The same study also showed that both red and white trout flesh (edible filet) contained very low levels of the analyte. Oikari et al. [1984] then developed a method to determine concentrations of free and conjugated resin acids in the bile of rainbow trout. Glucuronide and sulfate typically are conjugated to metabolic by-products and bodily contaminants in the liver, to help the body excrete them efficiently. Since the liver releases these conjugated species into bile, the bile becomes the best choice for measuring recent exposure of fish to aquatic pollutants. While free resin acids were extracted directly from the bile matrix, conjugated resin acids (e.g., dehydroabietic acid) were liberated from their conjugate

group using base hydrolysis before extraction. The bile contained over 99% of the conjugated forms (bound to glucuronide and sulfate groups) of resin acids. Miettinen et al. [1982], in a field study, showed that biological treatment of pulp and paper mill effluent reduced concentrations of resin acids in rainbow trout bile. Oikari [1986] conducted a fish field study using caged roaches in Finnish waters, and found increasing concentrations of resin acids and chlorophenolics in bile of fish that were placed closest to the pulp and paper mill. Oikari and Kunnamo-Ojala [1987] repeated the work using caged rainbow trout at the same sites, and found that the resin acids and chlorophenols were present in conjugated form in bile at levels of 95% and 92% of total extracted concentrations, respectively. These data supported an earlier laboratory experiment in which the resin acids quantified were present in >99% in conjugated form. Niimi and Lee [1992] determined that the half-life for resin acids in bile tissue is less than 4 days.

Söderström et al. [1994] compared acid, base, and enzymatic hydrolysis of conjugated chlorocatechols in the bile of goldfish. They found that 80-90% of chlorocatechols were conjugated to glucuronide sugar groups, and that 10-20% of conjugation was due to sulfate groups. They also discovered that neither acid nor base hydrolysis readily broke sulfate conjugates, so the addition of sulfatase would be needed for complete recovery of conjugated chlorophenolics.

Morales et al. [1992] developed a method using a combination of glucuronidase and sulfatase enzymes to break up the bile conjugates; followed by extraction, an ethylation procedure, and GC/MS for chemical analysis.

A field study was conducted downstream from a pulp and paper mill that was changing from elemental chlorine to chlorine dioxide bleaching, to determine the

concentrations of chlorophenolic compounds in whitefish and longnose sucker bile [Owens et al. 1994b]. They found that levels of chlorophenolics dropped in bile after the conversion from Cl_2 to ClO_2.

Fish bile analyses can be used to trace exposure of fish to many different compounds. Tavendale et al. [1996] found resin acid degradation products in the bile of goldfish. Also, Leppänen and Oikari [1999] measured resin acids and retene in the bile of perch and roach. Even highly lipophilic compounds like 2,3,7,8-tetrachlorodibenzo-p-dioxin and 2,3,7,8-tetrachlorodibenzofuran were quantitated in the bile of a number of species of fish exposed to bleached kraft mill effluent [Owens et al. 1994a]. Johnsen et al. [1995] demonstrated that resin acids were found at levels greater than 50 μg/g in the bile of rainbow trout exposed to effluent from a thermomechanical pulping mill.

Resin Acid Toxicity and Physiological Effects

Resin acids are known to be acute toxins to some aquatic fauna. Zanella [1983] conducted laboratory toxicity studies exposing bluegill, fathead minnows, and *Daphnia magna* to dehydroabietic acid. The LC_{50} (median lethal concentrations) values for the pH 7 toxicity studies involving these organisms were 6.4, 3.2, and 6.35 mg/L, respectively. This work also demonstrated that the LC_{50} for dehydroabietic acid decreases as the pH increases, thus increasing toxicity. This supported similar results collected from a study where researchers used paper mill effluent at different pH values to measure LC_{50} concentrations in rainbow trout [McLeay et al. 1979].

Nikinmaa and Oikari [1982] exposed rainbow trout to dehydroabietic acid, and found that blood pO_2, erythrocyte numbers, and blood pH all decreased as a result of the exposure. All of these parameters reverted back to normal levels after the fish were

moved back to tanks containing control water. The researchers noted that concentrations of UDP-glucuronyltransferase (UDP-GT), the enzyme responsible for glucuronide conjugation in the liver, were depressed after the same exposure period. Bogdanova and Nikinmaa [1998] also showed that lampreys exposed to dehydroabietic acid had lower red blood cell counts, and they experienced lower blood pH values. Oikari and Nakari [1982b] found that rainbow trout exposed to bleached kraft mill effluent experienced decreases of almost 70% in glycogen levels. They also reaffirmed the low levels of UDP-GT, which were found to cause intoxication jaundice. Bushnell et al. [1985] conducted a laboratory study and found that dehydroabietic acid breaks down red blood cells. Oikari et al. [1983] discovered that the minimum effective water concentration of dehydroabietic acid that caused physiological responses was 20 µg/L. Resin acids cause jaundice in rainbow trout because of insufficient glucuronide-conjugated bilirubin release into the bile, resulting in increased levels of free bilirubin in the liver [Mattsoff and Oikari, 1987]. Also, Oikari et al. [1988] showed that lake trout exposed to pulp and paper mill effluent had lower hemoglobin levels and reduced growth rates.

Zheng and Nicholson [1998] found in a laboratory study that dehydroabietic acid caused damage to nerve cells by mobilizing calcium found in intracellular stores, which facilitated excess neurotransmitter release. Using freshwater mussels exposed to kraft mill effluent, Burggraaf et al. [1996] found that resin acids reached a steady-state concentration in the organisms after 7 days. They also discovered that the approximate depuration half-life for resin acids was 3 days.

Resin Acid Fate and Remediation

Tavendale et al. [1997a,b] conducted a 264-day study to determine the fate of dehydroabietic acid in anaerobic sediment collected from waters receiving pulp and paper mill effluent. They found that the primary degradation product was tetrahydroretene, while dehydroabietin and retene were minor degradation products. Hall and Liver [1996] discovered that over 75% of all resin acids sorbed to suspended solids under both aerobic and anaerobic conditions, although sorption equilibration was faster in the aerobic study (12 hours), while it took 5 days for equilibration to be achieved in the anaerobic study. They also found that dehydroabietic acid sorbed the least of any of the resin acids tested. Dehydroabietic acid was found to degrade faster by photolysis in humic-free waters than in humic-containing waters [Corin et al. 2000]. The major degradation product in humic waters was dehydroabietin.

Morgan and Wyndham [1996] characterized bacteria isolated from pulp mill effluent, and measured the anaerobic degradation of resin acids using those bacteria. Martin et al. [1999] summarized the bacterial degradation of abietane resin acids using bacterial species endemic to paper mill effluent and other sources. They proposed aerobic degradation pathways of dehydroabietic acid, abietic acid, and palustric acid. Five bacteria species isolated from mill effluents were found to degrade abietane resin acids in 7 days, while pimarane resin acids showed only 25% degradation during the 7-day study [Bicho et al. 1995]. Wilson et al. [1996] isolated *Pseudomonas* bacteria species that were proficient in degrading isopimaric acid. Zhang et al. [1997] discovered that the ammonium ion aids in the anaerobic bacterial degradation of dehydroabietic acid.

Farrell et al. [1993] and Brush et al. [1994] both used Cartapip™, a product made from blue stain fungus, to degrade wood extractives during pulping processes. Both studies lasted 2 weeks, and they found that resin acid levels were reduced by 22% after treatment. Patoine et al. [1997] used a continuous aerobic activated sludge reactor that reduced resin acid concentrations in effluents, but the reactor was easily overloaded and the bacterial populations declined significantly. Guiot et al. [1998] attempted to use an anaerobic/aerobic activated sludge biotreatment reactor to degrade dehydroabietic acid and abietic acid, but this process also overloaded the reactor, and the bacterial populations declined significantly. A four-stage treatment process was designed by Zender et al. [1994] that included anaerobic and aerobic stages, and a natural lake. This treatment process showed that most abietane acids degrade faster under anaerobic conditions, while pimarane acids break down quicker under aerobic conditions. Also, this process removed over 95% of total resin acids present in the effluent.

Phytosterols

Phytosterols were not studied in pulp and paper mill effluents in earlier years because they are not acutely toxic at concentrations normally present in the effluents. However, chronic effects in the form of endocrine disruption have been studied using some phytosterols and their nonspecific metabolites. Marsheck et al. [1972] used a *Mycobacterium* species to degrade a phytosterol mixture to androstenedione and other steroidal compounds. Androstenedione, infamous for its use by professional athletes as a performance enhancer, is a phytosteroid and is hormonally active.

Denton et al. [1985] exposed mosquitofish to phytosterols degraded by *Mycobacterium smegmatis* and found masculinization of the female gonopodia. Howell

and Denton [1989] repeated this study and detailed the morphology of the affected mosquitofish gonopodia, counting rays and segments to further their conclusions of endocrine disruptive effects. Krotzer [1990] exposed mosquitofish to a different mixture of phytosterols and found morphological differences in the gonopodia, and also found that the treated female fish exhibited masculine behavior. The one thing missing from these three studies was chemistry. The phytosterol degradation products were not analytically measured, so the question remains as to what causes the masculinization effects. Hunsinger and Howell [1991] treated fish with androstenedione and found endocrine effects at minimum concentrations of 8 mg/L, orders of magnitude above what has been found in paper mill effluent (0.14 nM found in the Fenholloway River) [Jenkins et al. 2001].

Intact phytosterols are now being researched as possible endocrine disruptors. Lehtinen et al. [1999] reported that fish exposed to phytosterols spawned eggs that had lower hatchability and survivability. Also, they showed that phytosterols, mainly campesterol, are found in both the eggs and young fry of exposed adults. Tremblay and Van Der Kraak [1999] found that rainbow trout exposed to beta-sitosterol produced higher levels of vitellogenin, and lower concentrations of pregnenolone. Pregnenolone is an intermediate compound between cholesterol and progesterone. Awad et al. [1998] showed both reductase and aromatase inhibition in rats fed foods high in phytosterols.

Endocrine Disruption

Kendall et al. [1998] defined an endocrine disruptor as a compound that has the ability to alter the homeostatic status of hormones in their interactions with associated receptors. In previous studies conducted at the Georgia-Pacific Palatka Mill Operation

(PMO) by the University of Florida and the United States Geological Survey's (USGS) Florida Caribbean Science Center, some endocrine disruptive effects were observed [Sepulveda et al. 2003] in largemouth bass exposed to the discharged effluent mixture. Many compounds are present in pulp and paper mill effluent, and it is difficult to ascertain which chemical or mixture of chemicals could be responsible for endocrine disruption (ED).

Specific mechanisms for endocrine disruption are not well known. The first and strongest assumption is that hormonally active compounds will bind to estrogen receptors and inhibit or prohibit the intended protein from binding to it. The estrogen receptor is known to bind to a number of hormonally active compounds. Other mechanisms such as secondary inhibition by reaction with the intended protein are also possible. Antiestrogenic, estrogenic, antiandrogenic, and androgenic compounds all bind to receptors and stimulate a wide variety of responses.

Some mechanisms were found to cause enzyme inhibition. In particular, the enzyme aromatase, which converts testosterone to estradiol, can be inhibited, affecting sex determination in fish birds and reptiles. Kiparissis et al. [2001] has reported the presence of genistein, an isoflavonoid that is known to both bind to receptor sites and inhibit aromatase in pulp and paper mill effluent.

Objectives

The objectives of this study were to explore the biological uptake and fate of naturally occurring compounds produced by pulp and paper mills in higher

concentrations than found in the environment. Specifically, these studies were designed to:

1. Identify compounds that would serve as chemical markers for exposure of fish to effluent from pulp and paper mills, especially during mill process changes.

2. Examine the effects of different effluent concentrations of these compounds on the bile concentrations.

3. Examine the fate, kinetics, half-life, and metabolites of ß-sitosterol in pulp mill effluents derived from two different sources.

CHAPTER 2
MONITORING PHYTOSTEROLS AND RESIN ACIDS AS CHEMICAL MARKERS IN A LARGEMOUTH BASS REPRODUCTIVE EXPOSURE STUDY

Introduction

Our society is strongly dependent on paper and paper products, because they are integrated into almost every niche in our culture. Paper products such as newspaper, cardboard, car parts, and toilet paper, provide us, respectively, with information, packaging, transportation, and personal hygiene. The process of making paper and paper products produces many by-products that are emitted into effluents; many of which are organic compounds [Peterman et al. 1980, Suntio et al. 1988, and Judd et al. 1995].

Investigation of potential endocrine disruptive effects in largemouth bass at Georgia Pacific's Palatka Mill Operation (PMO) in Florida [Sepulveda et al. 2000 and 2003, and Quinn et al. 2003] led to the need to perform chemical exposure studies of the effluent emitted from the PMO into retention ponds, Rice Creek, and the St. John's River. In this study, three resin acids, isopimaric acid (IPA), dehydroabietic acid (DHA), and pimaric acid (PA), as well as four phytosterols, β-sitosterol, campesterol, stigmasterol, and stigmastanol were selected as chemical markers to study in the PMO effluent and bile from largemouth bass. These compounds were chosen because good analytical methodology was available, and both resin acids and phytosterols are of environmental concern, because they induce toxicity in aquatic organisms. Process changes occurred at the PMO during these studies, and these compounds were used as chemical markers to assess the effects of those changes. Some of the process changes include a new bleach

plant using chlorine dioxide, fixing sewer leaks from the brown stock washers, new condenser strips, and increased aeration of the effluent retention ponds.

Resin acids are known to decrease glycogen in the liver and increase plasma levels of glucose and lactate [McLeay et al. 1979]. Bleached Kraft mill effluent (BKME) has been found to cause inhibition of uridine diphosphate glucuronyltransferase (UDPGT) the enzyme responsible for glucuronidation in the liver; a phenomenon that increased during longer exposure times [Oikari and Nakari 1982b]. Their study also reported an increase in liver somatic index and the onset of jaundice. Resin acids induced acute hyperbilirubinaemia, jaundice, and inhibition of UDPGT in exposed rainbow trout [Mattsoff and Oikari 1987]. A mixture of resin and fatty acids with added chlorophenols was found to inhibit UDPGT and glutathione transferase enzymes in the liver [Oikari et al. 1988]. Resin acids do not remain long in the body of exposed fish during the depuration phase. A half-life of <4 days for resin acids was calculated after a 30-day exposure period and a 10-day depuration period [Niimi and Lee 1992]

Phytosterols are also sub-lethal toxins to aquatic fauna. A mixture of phytosterols induced inhibition of UDPGT (but only in females at the highest concentration), increased dose-dependent egg mortality, and smaller egg size in brown trout [Lehtinen et al. 1999]. The phytosterol β-sitosterol was found to decrease plasma levels of pregnenolone, an intermediate compound in the pathway between cholesterol and progesterone, in immature rainbow trout [Tremblay and Van Der Kraak 1999]. A study using the European polecat exposed to a mixture of phytosterols increased estradiol levels in both sexes and changed the thyroid ratio of T3/T4 [Nieminen et al. 2002]. One of the more striking studies showed that zebrafish exposed to a phytosterol mixture produced a

marked difference in sex ratios of offspring by changing from a male dominated population to a female dominated population [Nakari and Erkomaa 2003].

Bile analyses to determine exposure to organic compounds derived from paper mill effluents have become more common. Resin acids were measured in bile of rainbow trout in 3- and 20-day exposure studies [Oikari et al. 1984], while resin and fatty acids were measured in bile from lingcod [Morales et al. 1992]. Chlorophenolics, including chlorocatechols, were measured in the bile of sea perch [Soderstrom et al. 1994]. Chlorophenols, chloroguaiacols, chlorocatechols, chlorovanillins, fatty acids, and resin acids were analyzed from the bile of mountain whitefish and longnose sucker [Owens et al. 1994a]. Retene, a recalcitrant degradation product from the anaerobic metabolism of resin acids, was measured in the bile of roach and perch found downstream from a pulp and paper mill [Leppanen and Oikari 1999].

An extensive study was conducted to determine the uptake of resin acids in the tissues of trout [Oikari et al. 1982a]. Their results showed that resin acids were found primarily in blood plasma and bile, while the edible fish meat contained very little of these compounds. Further studies [Miettinen et al. 1982] determined that resin acids concentrated in the bile of trout following a 20-day exposure. These studies, and the fact that the plasma concentrations of resin acids from previous experiments [Sepulveda et al. 2003] were very low, while bile resin acid concentrations were very high, indicated a need for the studies of fish bile.

The objectives of this study were to measure the concentrations of selected resin acids and phytosterols in PMO effluent at different dilutions and in the bile of largemouth bass to determine chemical exposure, and to determine which compounds serve as the

best chemical markers. These data are compared to biological data from [Noggle et al. 2004] to clarify physiological effects found in exposed largemouth bass. The data are then compared to major process changes at the PMO that occurred during the conduct of these studies.

Methods and Materials

Site Description

Since 1947, Rice Creek, a tributary to the St. John's River has received the effluents from Georgia Pacific's PMO located in Palatka, FL. This mill has two bleaching lines (40% product) and an unbleached line (60% product), which together released an estimated 136 million liters of effluent/day before process changes and a reported 80 million liters of effluent/day after process changes. The pre-process change bleaching sequence for the PMO bleach lines is $C_{90}d_{10}$EopHDp and the post -process change sequence was DpEopDp, where Cd represents a mixture of chlorine (C) and chlorine dioxide (d) in proportions designated by subscripts, Eop is extraction with alkali (E), and with the addition of elemental oxygen (o) and hydrogen peroxide (p), H stands for hypochlorite, and Dp is chlorine dioxide with added hydrogen peroxide. The bleaching lines are used in the manufacture of paper towels and tissue paper, whereas the unbleached line produces mainly kraft bags and linerboard.

At the time of this study, the PMO effluents received secondary biological treatment for a reported 40-day retention time in four lagoons that were connected in series through a system of weirs. All lagoons were equipped with numerous aerators to help facilitate aerobic waste treatment. The treated effluent was released through a weir into a concrete chute from where it flowed through a lengthy earthen ditch into Rice

Creek, and thence to the St. John's River. Some oxygenated effluents are also released directly from the mill into Rice Creek at two different locations using elevated sprinklers.

In-situ Bass Exposure Study Design

In this study, largemouth bass were exposed for 56 days in both 2001 and 2002 to five different concentrations of biologically treated effluent, including 0, 10, 20, 40, and 80% dilutions. The 56-day exposure periods began during late winter when the largemouth bass started to become reproductively active. Adult largemouth bass were obtained from a fish farm (American Sportfish Hatcheries, Montgomery, Alabama), and transported to the USGS Florida Caribbean Science Center, Gainesville, Florida, where they were held in 0.04ha fish ponds until the start of the dosing experiment. After all fish were moved to Georgia-Pacific's PMO, they were acclimated in the test tanks for one week before dosing with mill effluent. At the PMO, fish were held outdoors in ten 1,500-L round, plastic flow-through tanks. Two additional 1,500-L tanks were used to create a head pressure for each of two treatments (well water control and effluent). Head tanks were held aloft on a 2.5-m tower. Water used for the control tanks and for effluent dilution was obtained from a well located in close proximity to the tank system. Well water was first pumped through a series of three 27,750-L pools containing biological media (sediment and aquatic vegetation), and then into the head tank. The larger pools were added to the design to increase the water quality since it was found that the well water contained low concentrations of iron, sulfides, and copper. A single, high volume, low-pressure air pump was used to aerate all tanks. In-line digital flow meters (ECOSOL®, Ontario, Canada) were set in each tank to control well water and effluent inputs, providing various effluent concentrations. Each exposure tank was initially

stocked with 60 bass and the fish were fed weekly with commercial fish pellets (Floating Fish Nuggets, Zeigler, Gardners, PA). The test system was designed to dilute pulp and paper mill effluent with treated well water at 10, 20, 40, and 80% effluent concentrations for 56 days to determine possible endocrine disrupting effects in largemouth bass.

Effluent Samples

Effluent samples were collected at least biweekly from each treatment level during the 56-day exposure study, extracted and analyzed to determine the concentrations of IPA, DHA, PA, β-sitosterol, campesterol, stigmasterol, and stigmastanol. On each sampling date, effluent from the tanks was collected just below the water surface in clean, 1-L amber bottles. After discarding the first fill and keeping the second fill, the pH was adjusted on some sub-samples to 10 with 2.5 N NaOH to stabilize resin acids and other sub-samples were adjusted to pH 2 using 5 N H_3PO_4 to stabilize phytosterols. Upon returning to the laboratory, the samples were stored at 4°C for up to 60 days prior to analysis.

Resin Acid Extraction

A 250-mL aliquot was taken from each sample and 10 mL of a citrate buffer (5.6 g in 100 mL) was added. All samples were fortified at 40 µg/L with a surrogate solution of methyl-o-methyl podocarpic acid to assess extraction and method efficiency. Each sample was adjusted to pH 4 with 8 M sulfuric acid and extracted three times with methyl-tert-butyl ether (MTBE); first with 60 mL, then twice with 40 mL. All emulsions were collected with the extracts and returned to the separatory funnel until they had dissipated. The extract was then concentrated to approximately 5 mL utilizing a Zymark Turbovap (Zymark Corporation, Hopkinton, MA).

Sample extracts were transferred, using a pasteur pipette, to 15-mL conical tubes with particular care to omit any water left in the flask. The tubes were placed in a water bath at 80°C until approximately 0.5 mL of liquid remained and the tubes were then removed and allowed to cool to room temperature (approximately 21-23°C). To each sample, 1 mL of isopropanolamine was added to trap free radicals and all solutions were mixed thoroughly for one minute. A 1-mL aliquot of triethyloxonium tetrafluoroborate (TEOTFB), an ethylation agent to derivatize target analytes, was added to each solution and again each sample was mixed thoroughly for one minute. A 1-mL aliquot of a saturated KCl solution was added and the sample was agitated for another minute. Each sample was extracted three times with hexane, first with 4 mL then twice more with 2 mL. A 250-µL aliquot of Ethanox 702™ [4,4'-methylene bis (di-t-butylphenol)] was added to each solution prior to concentrating the samples to 0.5 mL under a gentle stream of nitrogen to retard oxidation of the analytes. Methyl-o-methyl podocarpate was added as an internal standard and the samples were then analyzed by gas chromatography utilizing a mass spectrometer detector (GC/MS). This method is based on the current procedures used by NCASI to determine resin acid concentrations in aqueous samples [NCASI 1997].

Phytosterol Extraction

A 200-mL aliquot was taken from the sample containers and the pH was adjusted to 7 with a 50 mM pH 7 phosphate buffer. The samples were then extracted 4 times with 25 mL of MTBE. This extract was concentrated to 2-3 mL using a Zymark Turbovap (Zymark Corporation, Hopkinton, MA) and 20 mL of hexane was added to facilitate a solvent exchange. Each sample was then concentrated to 0.5 mL with nitrogen and

passed through sodium sulfate packed in a Pasteur pipette. The sodium sulfate was rinsed with 2-3 mL hexane and the sample was concentrated to 0.25 mL using nitrogen. A 0.25-mL aliquot of acetone was added to the extract along with 0.1 mL of n-methyl-n-(trimethylsilyl)-trifluoroacetamide (MSTFA) and the sample was capped and allowed to derivatize for at least one hour at room temperature. The samples then sat at least one hour before they were transferred to 0.8-mL amber autosampler vials in which a semi-volatile internal standard mix was added as internal standard prior to analysis by GC/MS. The compound d12-perylene was used as the internal standard for quantitation purposes.

Bile Samples

Bile samples were collected on days 0, 28, and 56 of the exposure study. Gall bladders were carefully removed from the fish and drained into a conical freezer vial and samples were put on ice until arrival at the laboratory where they were stored at –80°C until analysis.

Bile samples were thawed and transferred from freezer vials to culture tubes using a syringe to carefully measure the volume. One mL of pH 4 acetate buffer was added to each sample in addition to the enzymes glucuronidase and sulfatase, and 6-bromo-2-naphthol-ß-glucuronide in methanol as a surrogate [Morales et al. 1992]. The culture tubes were placed in an incubator at 37°C for 10-13 hours to facilitate the hydrolysis of glucuronide and sulfate conjugates. Each sample was extracted three times with 4 mL MTBE and the pooled extract volume was amended to 12 mL. Six mL, each, were removed and placed in a separate tube for analysis of phytosterols and resin acids.

The first 6-mL aliquot, taken for phytosterol analysis, was evaporated to dryness using a gentle stream of N_2. A 0.5-mL aliquot of 1:1 hexane acetone was added to each

sample along with 0.1 mL MSTFA and the centrifuge tube was capped and agitated for 1 minute. The samples sat at least one hour before they were transferred to 0.8-mL amber autosampler vials in which a semi-volatile internal standard mix was added as internal standard prior to analysis by GC/MS. The compound d12-perylene was used as the internal standard for quantitation purposes.

The other half of the sample extracts, used for resin acid analysis, was transferred to 15-mL conical tubes with care taken to exclude any water. The tubes were placed in a water bath at 80°C and heated until 0.5 mL of liquid remained. The tubes were then removed and allowed to cool to room temperature. Prior to analysis, 1 mL of isopropanolamine was added to each sample and all solutions were mixed thoroughly for one minute. One mL of triethyloxonium tetrafluoroborate, an ethylation agent to derivatize target analytes, was added to each solution and again, each sample was mixed thoroughly for 1 min. A 1-mL aliquot of a saturated KCl solution was added to each sample and the samples were again agitated for 1 min. Each sample was extracted three times with hexane, first with 4 mL, then twice more with 2 mL, each. A 250-µL aliquot of Ethanox 702™ [4,4'-methylene bis (di-t-butylphenol)] was added to each solution before concentrating the sample volume to 0.5 mL under a gentle stream of N_2. Methyl-O-methyl podocarpate was added as an internal standard before analysis by GC/MS.

Results and Discussion

Resin acid concentrations in effluent samples showed dose dependent relationships (Figures 2-1 & 2-2) based on the test system designed to deliver the different target effluent percentages. The only exception to this was between 20% and 40% effluent in 2001, which was likely due to faulty flow valves at the 40% dilution.

The process changes produced a marked drop in all resin acid effluent concentrations. The DHA effluent concentrations between 2001 and 2002 appear in Figure 2-3. The most significant process changes that would effect resin acid concentrations would be fixing leaks in the brown stock washer sewer lines and the addition of more aerators in the retention ponds. In 1999, this same system had an average of 6.42 mg/L of IPA for the 80% treatment level with spikes as high as 15.6 mg/L [Sepulveda et al. 2003]. The IPA concentrations for the 80% treatment levels averaged 0.12 mg/L in 2002. Surrogate recoveries for ethyl-o-methyl podocarpate in effluent were 106% with a standard deviation of 11% in 2001, and 111% with a standard deviation of 8% in 2002. The linear range from the GC/MS analysis of resin acids was 2-50 mg/L. Phytosterols were only recorded in 100% effluent in 2001, and the concentrations in pure effluent from 2002 were all found to be below the detection limit of 20 μg/L in the first 3 sampling events. The phytosterol β-sitosterol was, by far, the most abundant compound with a concentration of 1.07 mg/L. Average concentrations for stigmastanol, campesterol, and stigmasterol were 0.14, 0.08, and 0.08 mg/L, respectively. The linear range from the GC/MS analysis of phytosterols was 2-40 mg/L. The surrogate recoveries of cholesterol in effluent samples averaged 116% with a standard deviation of 17%.

Resin acid concentrations in bile were not dose dependent in either 2001 or 2002 (Figures 2-4 & 2-5). This was also observed in a related study conducted at the PMO [Sepulveda et al. 2003]. In the 10-20% effluent concentrations for both years, the concentration of DHA in bile was much higher than PA and IPA, but concentrations were similar for all three compounds at the higher effluent dilutions. Most resin acid concentrations were depressed in the higher effluent concentrations. There was a sizable

decrease in resin acid bile concentration levels after process changes. The difference in DHA bile concentrations between 2001 and 2002 is depicted in Figure 2-6. Phytosterol concentrations in bile exhibited a more marked decline than resin acids as effluent concentration increased, especially above 20% effluent (Figure 2-7). Campesterol was, by far, the most abundant phytosterol quantified in bile. This phenomenon agrees with previous work conducted on phytosterols in bile [Lehtinen et al. 1999]. All phytosterol concentrations in bile, except campesterol (Figure 2-8) dropped below detection limits (3 μg/mL) following process changes at the PMO mill.

Treated BKME has been shown to inhibit UDPGT in trout [Oikari and Nakari 1982b], which would decrease concentrations of organic compounds excreted in bile and cause these compounds to pool in liver, plasma, and other tissues. A similar study [Oikari et al. 1983] observed inhibition of UDPGT and the onset of jaundice. A field study [Oikari and Kunnamo-Ojala 1987] showed that UDPGT concentrations increased in fish with distance from the BKME mill discharge point. A resin acid mixture induced acute hyperbilirubinaemia, jaundice, and inhibition of UDPGT in exposed rainbow trout [Mattsoff and Oikari 1987].

Compounds other than resin acids might be responsible for the inhibition of organic compound secretion in bile. Genistein, an isoflavone and aromatase inhibitor, has been found in BKME effluent [Kiparissis et al. 2001]. Genistein is responsible for inhibition of the UDPGT and the sulfotransferases SULT1A1 and SULT2A1 in rat livers [Mesia-Vela and Kauffman 2003]. Another study using rats demonstrated depressed excretion of gemifibrozil after exposure to genistein [Lucas et al. 2003]. These

enyzmatic pathways are basically the same in most vertebrates, so mammalian data likely applies to fish [Margaret James personal communication 2004].

Nonylphenol oxylates are common constituents of surfactants used in the pulp an paper industry [Berryman et al. 2004], and these compounds were found to inhibit, p-glycoprotein, a membrane transfer protein, in channel catfish [Kleinow et al. 2004]. Nonylphenols are biodegradation products of nonylphenol oxylates [Giger et al. 1984]. Nonylphenols are weak estrogens that bind to 17β-estradiol receptors [White et al. 1994]. While it is unlikely that nonylphenols are responsible for androgenic effects in mosquitofish, the role they could play as endocrine disruptors in pulp and paper mill effluent should be explored.

In conclusion, resin acids found in bile are appropriate chemical markers of fish exposure to pulp and paper mill effluent. Phytosterols are a poorer choice as chemical markers due to lower concentrations relative to method detection limits. Bile concentrations of organics discharged from pulp and paper mills are better used as qualitative indicators of exposure due to the lack of clear dose-response relationships. Process changes decreased resin acid and phytosterol concentrations in effluent and the bile of exposed fish.

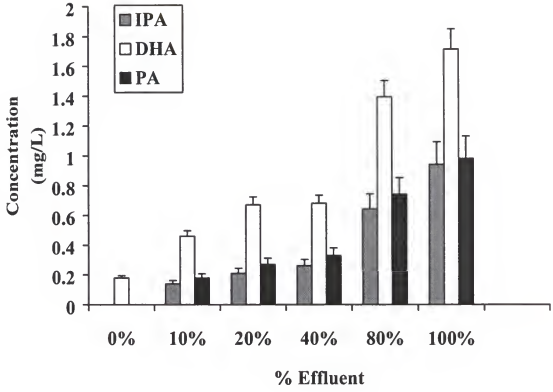


Figure 2-1. Resin acid concentrations in effluent for 2001 with standard error bars.

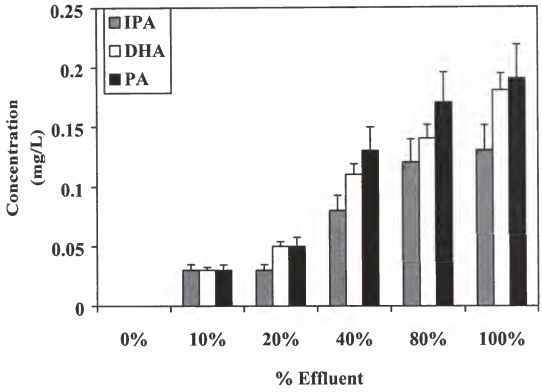


Figure 2-2. Resin acid concentrations in effluent for 2002 with standard error bars.

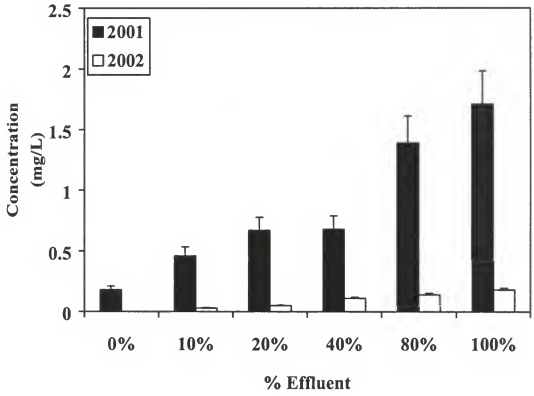


Figure 2-3. DHA concentrations in effluent for 2001-2002 with standard error bars.

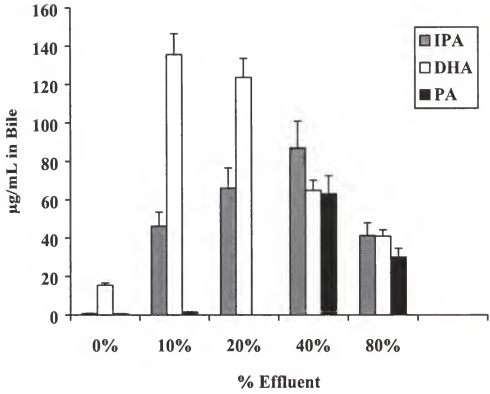


Figure 2-4. Resin acid concentrations in bile for 2001 with standard error bars.

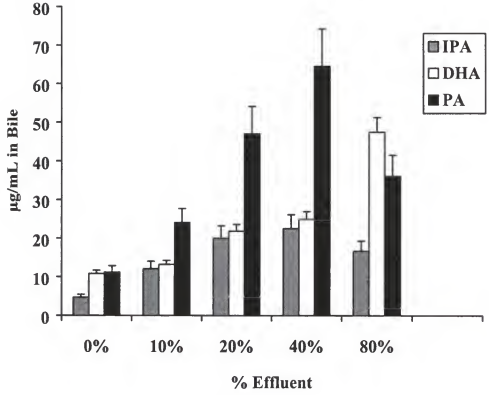


Figure 2-5. Resin acid concentrations in bile for 2002 with standard error bars.

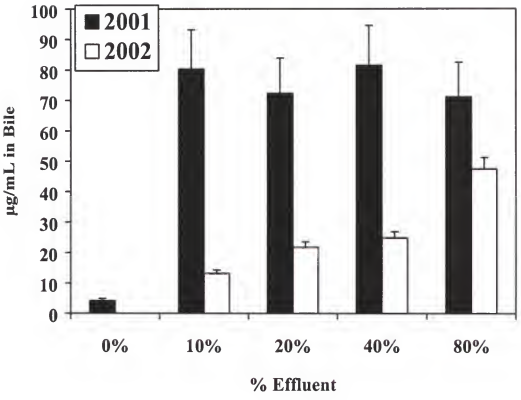


Figure 2-6. DHA concentrations in fish bile from 2001-2002 with standard error bars.

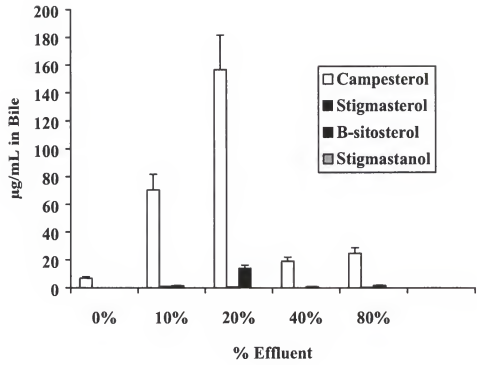


Figure 2-7. Phytosterol concentrations in fish bile for 2001 with standard error bars.

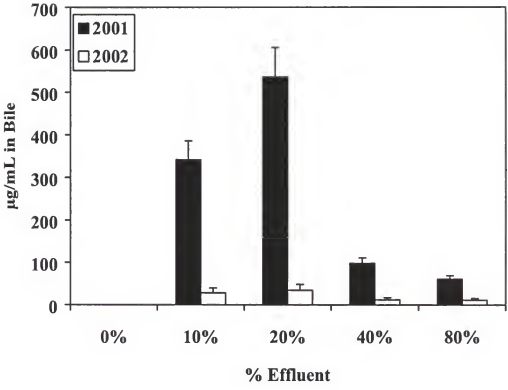


Figure 2-8. Campesterol concentrations in bile from 2001-2002 with standard error bars.

CHAPTER 3
DEGRADATION OF ß -SITOSTEROL IN PULP AND PAPER MILL EFFLUENTS

Introduction

Phytosterols are a common component in pulp and paper mill effluents [Peterman et al. 1980 and Suntio et al. 1988]. Various studies have shown that phytosterols elicit sub-lethal effects in exposed aquatic organisms. A mixture of phytosterols increased dose-dependent egg mortality, and smaller egg size in exposed brown trout [Lehtinen et al. 1999]. The phytosterol ß-sitosterol induced higher vitellogenin concentrations, and decreased plasma cholesterol and pregnenolone, and intermediate compound between cholesterol and progesterone, concentrations in immature rainbow trout [Tremblay and Van Der Kraak 1999]. Zebrafish exposed to phytosterol mixtures including ß-sitosterol had induced higher levels of vitellogenin indicating the onset of reproduction and a reversal of sex ratios from a male dominated population to a female dominated population [Nakari and Erkomaa 2003].

These sub-lethal effects can be solitary or synergistic. One synergistic example shows that when pulp and paper mill effluents contain both resin acids and phytosterols, sex steroids can be altered. Resin acids inhibit uridine diphosphate glucuronyl transferase (UDPGT) production [Oikari and Nakari 1982b], which causes an increase in the amount of phytosterols circulating in the blood plasma and other tissues, because they are not being excreted in bile. Phytosterols are widely known to decrease the circulating concentration of cholesterol. The decreased cholesterol level results in lower amounts of circulating androgens, because they are all derived from the conversion of cholesterol to

pregnenolone, and then progesterone. Progesterone is converted to testosterone, corticosteroids, and aldosterone, which circulate throughout the body performing many different endocrine functions (Figure 3-1).

Much more attention has been paid to degradation products of sterols. The bacteria *Mycobacterium sp.* has been found to degrade sterols by dealkylating the side chains under laboratory conditions, leaving the steroidal ring structure intact to transform into various androgenic compounds including androstenedione [Marsheck et al. 1972, Ambrus et al. 1995, and Lamb et al. 1998]. This led to a number of experiments designed to explain why female mosquitofish were masculinized while being exposed to pulp and paper mill effluent [Howell et al. 1980]. Female mosquitofish exhibited masculinized anatomical behavior when exposed to a mixture of phytosterols dosed with active *Mycobacterium smegmatis* [Denton et al. 1985 and Krotzer 1990]. A similar study exposed female mosquitofish to a mixture of the phytosterol stigmastanol and *Mycobacterium smegmatis*, which induced masculinization [Howell and Denton 1989]. The common thread in all of these studies was that no analytical chemistry was conducted on the phytosterol/bacterial mixtures, leaving only the unproven hypothesis that the causative agent(s) were androgens formed from degraded phytosterols. These assumptions were bolstered when androstenedione was detected at low concentrations in the Fenholloway River in northern Florida [Jenkins et al. 2001], which is one of the field sites that provided source water for this study.

All sites chosen for bacterial seed are in North Florida and both are Kraft mills. The bacterial seed consists of the consortium and abundance of microorganisms present in each sample. Sites impacted by pulp and paper mills are expected to have greater

diversity and numbers of bacteria, because of the large concentrations of organic compounds present and acting as electron donors for microorganisms.

The first site was located on the Fenholloway River, near Perry, Florida, which receives 174 million liters per day of effluent from Buckeye Florida, a dissolving Kraft pulp mill. This mill uses only slash pine because it contains long cellulose fibers that produce high-grade cellulose products. Effluent from this mill is treated for 5 days in 13 retention ponds (11 are aerated) and then released into the Fenholloway River for an average 2.5-day residence time, before emptying into the Gulf of Mexico. The second site was Rice Creek, a tributary of the St. John's River, which has received the effluents from Georgia Pacific's Palatka Mill Operation (PMO) located in Palatka, Florida, since 1947. This mill has two bleaching lines (50% product) and an unbleached line (50% product), which together release an average 95 million liters of effluent/day. Effluent from the PMO is piped to a series of aerobic ponds that have a reported 40-day retention time. Previous field studies at the PMO and its receiving waters have shown endocrine disruptive effects in aquatic organisms [Bortone and Cody 1999, Sepulveda et al. 2000, and Sepulveda et al. 2003].

The objectives of this study were to (a) assess the environmental fate of ß-sitosterol in pulp and paper mill effluent under aerobic and anaerobic conditions; (b) determine reaction rates and kinetics; and (c) to identify any metabolites.

Methods and Materials

Effluent Sampling

In January 2004, 12 L of water was collected for a preliminary experiment from the Fenholloway River at the US 19 bridge, 0.4 miles downstream from the Buckeye

Florida pulp mill. Water quality parameters including pH, dissolved oxygen, conductivity, temperature and salinity were recorded before the samples were taken to the United States Geological Survey (USGS) facility in Gainesville, Florida and incubated in darkness at 30°C for 14 days prior to study initiation.

In March 2004, 12 L of water was collected for a more definitive study at the same location used for the preliminary experiment in January 2004, and near the US 27 bridge 7.7 miles upstream from the Buckeye Florida pulp mill. Additional 12-L samples were collected from Rice Creek at the State Road 100 bridge (upstream reference site), and at the first aerator downstream where effluent from the PMO enters Rice Creek. These samples were taken to the USGS facility in Gainesville, Florida and incubated in darkness for 10 days prior to study initiation.

In April 2004, duplicate 10-L effluent samples were collected for a different degradation study from the effluent-impacted sites at Rice Creek and the Fenholloway River used in the previous studies. These samples were taken to the USGS facility in Gainesville, Florida and incubated in darkness for 7 days prior to study initiation. All incubation periods were conducted to bolster the bacterial seed collected from the sampling sites.

Compound Information

A radiolabelled test compound, ^{3}H-ß-sitosterol (10 mCi with a specific activity of 38 Ci/mmol) was obtained from New England Nuclear, a division of Perkin-Elmer Life Sciences, Inc. (Wellesly, MA), and stored at –80°C for 10 months. The purity was found to be less than 70% after this storage period, and extensive purification using HPLC/fractionation methodology was required before conducting the environmental fate

studies. After the purification process, the ^{3}H-ß-sitosterol purity was improved to 96.6% for the preliminary study and 93.3% for the definitive study.

Study Design

The preliminary study design integrated continuous gas flow into dosed water/effluent samples incubated in the dark at 30°C. Either nitrogen or compressed air was bled into a test system at 1-2 mL/min, controlled by a Swagelock® stainless steel needle valve and measured with an in-line flow meter. The preliminary test system began with ~200 mL of DI water in a 250-mL gas-washing bottle, which was added to saturate the gas; and ensure that the duplicate reaction vessels per system did not lose volume. The reaction vessel was a 250-mL gas-washing bottle filled with 200 mL of sample, which was nominally dosed with ^{3}H-ß-sitosterol at 10,000 dpm/mL. The reaction vessel was vented to a bed of activated carbon to prevent potential airborne contamination from loss of tritiated compound. The definitive study design differed from the preliminary design in two ways. First, only compressed air was used, because only aerobic conditions were desired, and second, a 250-mL gas-washing bottle filled with 100 mL of 10% ethylene glycol in water was added behind the reaction vessel to trap possible volatile compounds.

The non-radiolabelled ß-sitosterol aerobic degradation study was conducted to determine any metabolic products by GC/MS. One of the duplicate 10-L samples was dosed with 25 mg of ß-sitosterol (resulting concentration was 2.5 mg/L), while the other samples was not dosed. Both samples were incubated at 30°C in darkness and constantly mixed using a magnetic stir plate. The samples were taken from the incubator after and 10-11 days and added to a continuous extractor apparatus where they were extracted for

approximately 18 hours using methylene chloride. The methylene chloride extracts were concentrated to 1 mL using a Zymark Turbovap (Zymark Corporation, Hopkinton, MA).

Study Sampling

Samples for the preliminary study were taken from each reaction vessel at hour 0, 43, 116, 211, 308, and 360, and promptly refrigerated until analysis. Samples taken in the definitive study at hours 0, 19.5, 69, 164, 260, 500, and 717 were also refrigerated until analysis. Sampling consisted of carefully opening the reaction vessel, taking a 1-mL aliquot using an Eppendorf 1-mL adjustable pipette, and adding it to a 7-mL scintillation vial.

Instrumental Analysis

A 90-µL aliquot of each sample was injected into an HPLC system that included a Perkin-Elmer LC250 pump operating at 1 mL/min that was followed by a Perkin-Elmer LC-95 UV/Vis detector set at 205 nm, and finally, a Gilson FC-203B fraction collector. The stationary phase was a Supelco Discovery C8, 4.6 x 150 mm, with a 5-µm particle size, and the isocratic mobile phase was 80:20 acetonitrile:water, (v:v), which was degassed using helium. Forty-four 1-min fractions were collected in 7-mL scintillation vials, 5.5 mL of Scintverse® LC scintillation cocktail was added to each, and the sample were analyzed with a Packard liquid scintillation counter.

Results and Discussion

The preliminary study produced valuable information that shaped the scope of the definitive study. The first major finding showed that ß-sitosterol degraded much faster under aerobic conditions, which led to the definitive study being conducted totally under aerobic conditions. The half-life of ß-sitosterol in effluent under aerobic conditions was

calculated as 6-10 days. The degradation kinetics followed first-order behavior with r^2 values of 0.92 and 0.97 (Figure 3-2) for the two replicates. The half-life in the anaerobic system was 72-144 days with r^2 values of 0.44 and 0.07 for the two replicates, suggesting that anaerobic degradation was not first-order, and aerobic degradation was the primary pathway in pulp mill effluent.

The definitive study demonstrated that effluent samples from both receiving waters in Rice Creek and the Fenholloway River, facilitated the aerobic degradation of ß-sitosterol. Both reference samples proved to degrade this phytosterol as well, but at a slower rate. Effluent samples from Rice Creek demonstrated a degradation half-life of 22-24 days with r^2 values of 0.932 and 0.860 for the two replicates (Figure 3-3). The Fenholloway River effluent samples showed a ß-sitosterol degradation half-life of 24-29 days with r^2 values of 0.933 and 0.833 (Figure 3-4). Reference samples from Rice Creek had an aerobic degradation half-life of 32-41 days and lower r^2 values of 0.667 and 0.779 (Figure 3-5), while the Fenholloway River reference samples showed a degradation half-life of 32-36 days for ß-sitosterol with r^2 values of 0.897 and 0.891 (Figure 3-6). Radioactive compounds trapped in the 10% ethylene glycol in water mixture were barely above background levels, demonstrating that there was little loss of radioactivity to volatility.

The non-radiolabelled study did not yield the metabolites 4-androsten-3,17-dione and 1,4-androstadiene-3,17-dione, although a tentative GC/MS library match was obtained for androsteneone. Many unknown compounds containing a steroidal structure were observed, but standards were not available to obtain tentative identifications. All phytosterols were found in dosed and reference samples. Two compounds, nonylphenol

and octylphenol, degradation products of commercial surfactants [Giger et al. 1984], were detected in abundance in the Rice Creek samples. This was a result of sampling near the liquid oxygen injection system that also adds surfactants to the treated effluent. Nonylphenol binds to estrogenic receptors and is considered to be a weak endocrine disruptor [White et al. 1994], but it is, most likely, not the cause of the androgenic found in mosquitofish exposed to pulp and paper mill effluents.

This study demonstrated that microorganisms responsible for the aerobic metabolism of ß-sitosterol are present in the two effluent impacted streams used in this study. Many exposure studies concentrated on *Mycobacterium smegmatis* as the primary species of bacteria responsible for side chain dealkylation of sterols, and this species does produce that reaction [Marsheck et al. 1972, Ambrus et al. 1995, and Lamb et al. 1998]. It is not likely that this species is responsible for ß-sitosterol degradation in these waterways, especially in the Fenholloway River with its low DO levels, because *Mycobacterium smegmatis* either goes dormant or dies under hypoxic and anaerobic conditions [Dick et al. 1998]. Other microorganisms have been found that dealkylate sterol side chains to produce steroidal compounds. The blue-green algae *Chlamydomonas reinhardtii* has been reported to induce this reaction [Giner and Djerassi 1992]. *Arthrobacter oxydans* has also been found to dealkylate sterol side chains [Dutta et al. 1992]. *Rhodococcus sp.* produces cholesterol oxidase, which dealkylates sterol side chains [Elalami et al. 1999] and the 3-kesteroid-Δ^1-dehydrogenase enzyme that is first to cleave the steroidal ring [van der Geize et al. 2000]. Other bacteria are known to cleave the ring structures of sterols and steroidal compounds [Mahato and Garai, 1997], but the resulting metabolites have been of less interest because of the lack of steroidal properties.

It is possible that masculinization of mosquitofish has been induced by steroidal compounds and androgenic metabolites produced from aerobic phytosterol degradation. Another possible mechanism, the inhibition of aromatase activity, was studied in mosquitofish from the Fenholloway River, and this study showed that this pathway of masculinization was not probable [Orlando et al. 2002]. Further investigations of in-situ aerobic microbial metabolites should be explored to better explain this phenomenon.

In conclusion, ß-sitosterol degrades under aerobic conditions in both pulp mill effluent and in natural streams used as reference waters. Preliminary aerobic degradation studies determined the half-life of ß-sitosterol under aerobic conditions to be 6-10 days. The half-life range of the effluent samples was 22-28 days for both effluent dominated streams. The half-life range of the two reference samples was 32-41 days. This aerobic degradation process follows first-order reaction rate kinetics. Changes in the bacterial seed collected on different days contributed to the difference in estimated half-life calculations. The most publicized aerobic microbial degradation products from ß-sitosterol in laboratory studies, 4-androsten-3,17-dione and 1,4-androstadiene-3,17-dione [Marsheck et al. 1972], were not detected as metabolites in this study.

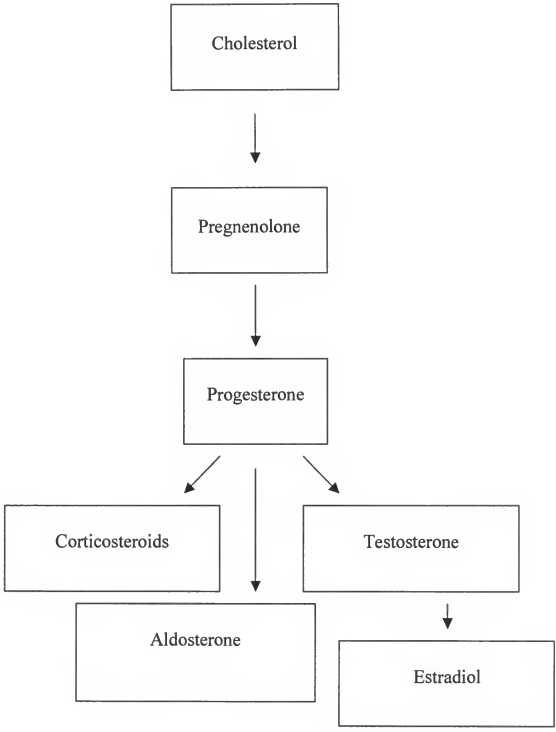


Figure 3-1. Endocrine Pathway in Vertebrates

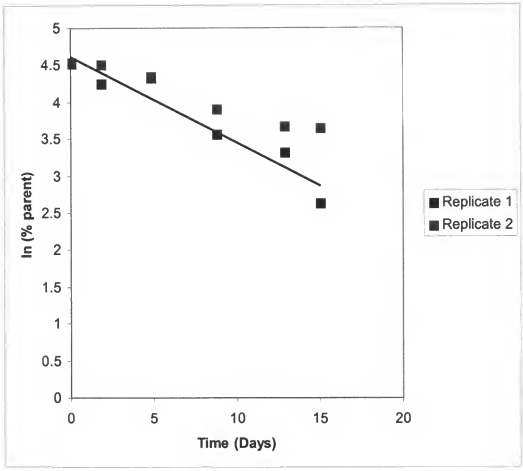


Figure 3-2. Fenholloway River effluent half-life curves for β-sitosterol from the preliminary study.

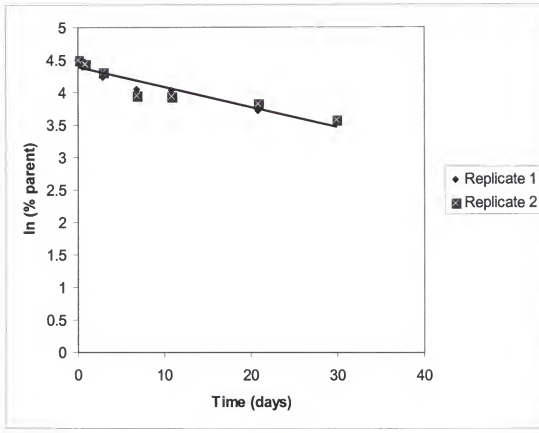


Figure 3-3. Rice Creek effluent half-life curves for β-sitosterol.

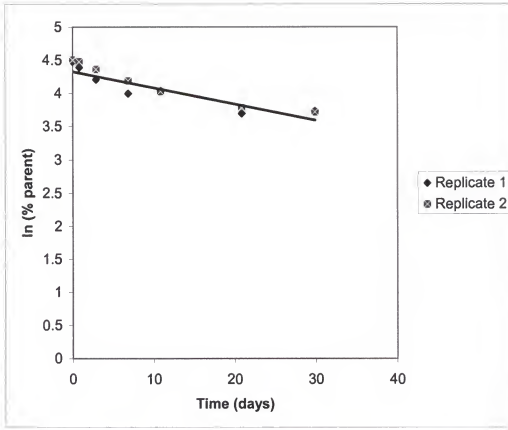


Figure 3-4. Fenholloway River effluent half-life curves for β-sitosterol.

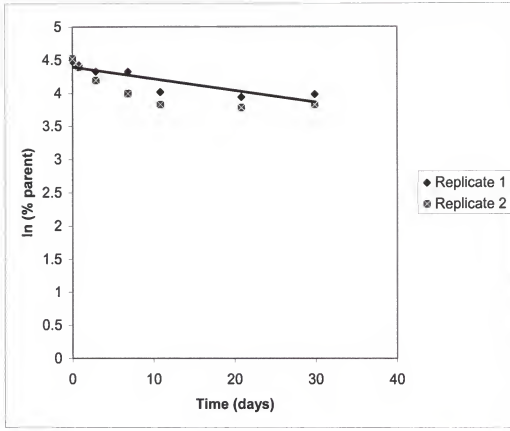


Figure 3-5. Rice Creek reference site half-life curves for β-sitosterol.

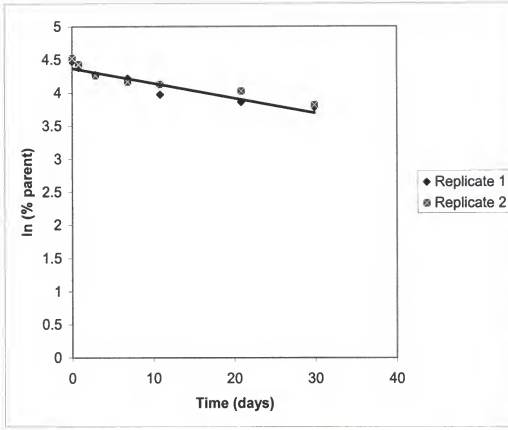


Figure 3-6. Fenholloway River reference site half-life curves for β-sitosterol.

CHAPTER 4
SUMMARY, CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

Summary

Pulp and paper mills emit effluents that contain many organic compounds. Early studies focused on acutely toxic and chlorinated compounds as the primary targets contributing to the impact of the effluents on organisms in receiving waters. That research led to different mill process changes such as eliminating elemental chlorine bleaching and adding secondary (biological) treatment that contributed to a less toxic discharge. Much of the last decade of research in pulp and paper effluents has shown that both large and small concentrations of different naturally occurring extractive compounds derived from wood pulping have induced sub-lethal effects in exposed organisms.

The studies conducted for this dissertation have sought to advance knowledge in the fields of aquatic toxicology, pulp and paper mill effluents, and environmental and analytical chemistry. Largemouth bass had not been used previously for these types of experiments, so that there were no models on which to base this work. Biological indices like the liver somatic index, gonadal somatic index, and circulating sex steroids were developed by wildlife biologists, and in this research, effluent and bile analyses were added to develop the chemistry for large-scale field studies. Correlation of biological and chemical data must be considered when running fish toxicity tests. Collaborative efforts showed that endocrine disruption, a type of sub-lethal toxicity, was observed in both the liver and in circulating sex steroids.

The microbial degradation studies involving the phytosterol ß-sitosterol provided valuable information. After degradation studies were conducted in both aerobic and anaerobic systems, it was clear that aerobic microbial degradation in effluent was the fastest pathway for phytosterol breakdown. The aerobic degradation process generally followed first-order kinetics and demonstrated a relatively short half-life, while the anaerobic degradation did not follow first-order kinetics and had a much longer half-life of the two systems. Both aerobic reference samples upstream from the two experimental field sites induced ß-sitosterol degradation, which indicated that microbes were present in natural, non-impacted systems capable of degrading phytosterols. The most well known aerobic microbial degradation products from ß-sitosterol, i.e. 4-androsten-3,17-dione and 1,4-androstadiene-3,17-dione, were not detected as metabolites in this study.

Conclusions

The following conclusions are drawn as they relate to the research performed to meet this study's objectives:

- Process changes at Georgia-Pacific's Palatka Mill Operation resulted in decreased concentrations of resin acids and phytosterols in effluent and the bile of exposed largemouth bass.
- Resin acids are good qualitative chemical markers in fish bile for exposure to pulp and paper mill effluents.
- Of the phytosterols, only campesterol is useful as a chemical marker in fish bile for exposure to pulp and paper mill effluents.
- ß-sitosterol degrades aerobically in streams that contain pulp and paper mill effluents.
- The half-life range of ß-sitosterol in streams containing pulp and paper mill effluents under dark, aerobic conditions at 30°C was 22-28 days.
- The aerobic degradation of ß-sitosterol in aerobic streams containing pulp and paper mill effluents generally followed first-order kinetics.

Recommendations for Future Work

The following recommendations are made to further the knowledge in the field of environmental chemistry related to pulp and paper mills:

- New analytical methods using liquid chromatography/mass spectrometry should be developed to analyze the many classes of wood extractive compounds present in pulp and paper mill effluents and their receiving waters.
- Chemical ionization/mass spectrometry should be used on these samples to determine molecular weights of unknown and tentatively identified compounds.
- A number of organic compounds derived from different classes of wood extractives need to be studied in the bile of exposed organisms to determine if molecular weight, chemical structure, or a mixture of both, dictates which are inhibited first and most in bile excretion.
- Exposure studies that use fish bile should include analyses for UDPGT to monitor liver dysfunctions.
- The ß-sitosterol degradation study should be performed using a ^{14}C-radiolabelled molecule to obtain a mineralization rate.
- Multiple samples from the process streams of the Buckeye mill leading to the salt water wedge in the Fenholloway River should be collected and degradation studies conducted to assess the role of salinity in affecting the viability of microbial populations responsible for compound fate.

- Aerobic and anaerobic degradation studies using ^{14}C-radiolabelled molecules should be conducted on representative compounds of different plant-derived chemical classes, especially those with multiple ring structures similar to steroidal compounds.
- All of these fate studies should be conducted in 3-L flasks to expose compounds to a larger population of microorganisms.
- Microbiological techniques should be developed to better assess the bacterial population in pulp and paper mill effluents.
- Fate and reference studies using non-radiolabelled compounds should be conducted by extracting large volumes of stream water and effluent (>50 L) to assess the identity compounds present in low concentrations.
- Whenever a metabolite with a steroidal structure is identified, largemouth bass and mosquitofish should be exposed to that compound to determine endocrine disruptive effects.

APPENDIX A
CHEMICAL STRUCTURES OF COMPOUNDS ANALYZED IN THIS STUDY

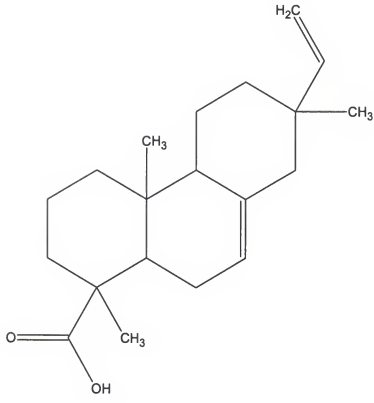


Figure A-1. Structure of isopimaric acid.

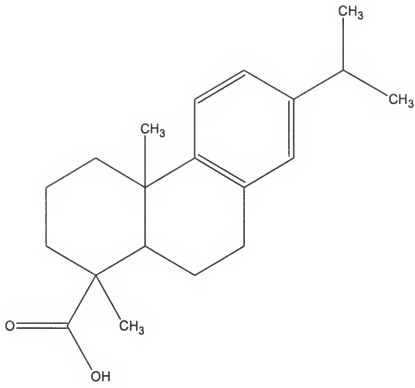


Figure A-2. Structure of dehydroabietic acid.

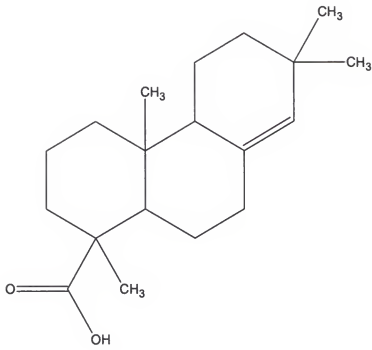


Figure A-3. Structure of pimaric acid.

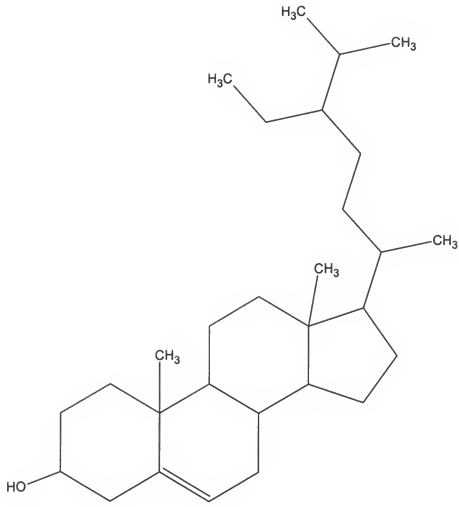


Figure A-4. Stucture of β-sitosterol.

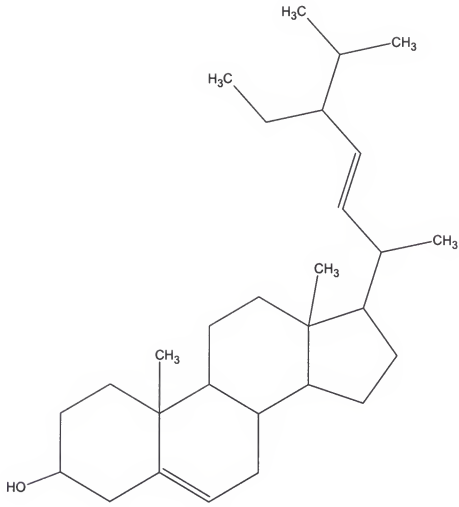


Figure A-5. Structure of stigmasterol.

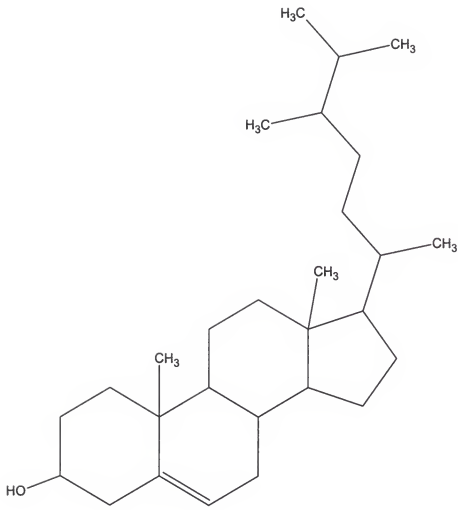


Figure A-6. Structure of campesterol.

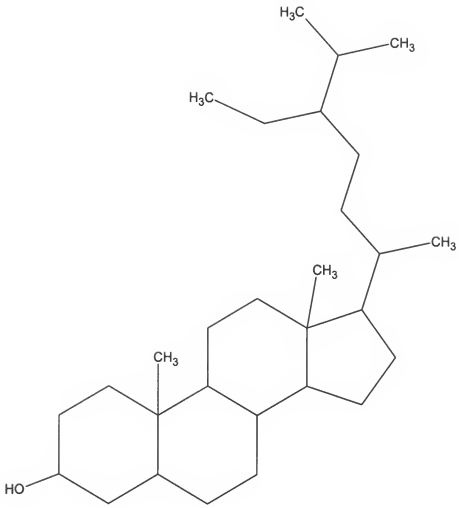


Figure A-7. Structure of stigmastanol.

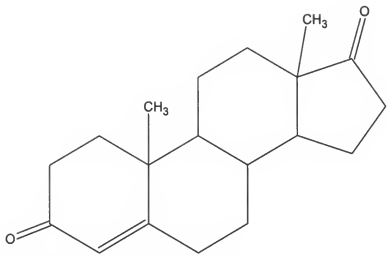


Figure A-8. Structure of androstenedione.

APPENDIX B
CALCULATING A DEGRADATION REACTION HALF-LIFE FROM RAW DATA

The half-life of a degradation reaction is calculated from the percent parent molecule versus time. The following procedure highlights each step towards correctly estimating the half-life of a reaction.

- Determine the retention time of the radiolabelled parent compound in an HPLC system.
- Total the DPMs from the peak that corresponds to the parent compound and divide by the total DPMs in the histogram to calculate the percent parent compound.
- Calculate the natural log of percent parent compound.
- Plot the ln (% parent compound) vs. time (sampling schedule).
- Calculate the slope (k) of the resulting curve, which is the reaction rate constant.
- Calculate the ln (2)/k to get the half-life of the reaction.

Example calculation: Samples were taken at hours 0, 19.5, 69, 164, 260, 500, and 717. The percent parent for each was 91.1, 87, 68.8, 57.1, 56.3, 41.2, and 34.3, respectively. The ln (% parent) values were 4.51, 4.47, 4.23, 4.04, 4.03, 3.72, and 3.54, respectively. This created a function with a rate constant of 0.0013. The final calculation of ln 2/k (k=0.0013) yields a half-life of 533 hours or 22.2 days.

APPENDIX C
RAW DATA INCLUDING MASS SPECTRA, CHROMATOGRAMS, HISTOGRAMS, AND TABLES

This appendix contains raw data and tables from chapters 2 and 3. The GC/MS data will be presented as spectra; total ion current (TIC) plots, and extracted ion chromatograms (EIC). Chapter 2 data tables will be followed by chapter 3 tables, histograms, and GC/MS data.

Table C-1. 2001 isopimaric acid effluent concentrations (all values in mg/L).

	0%	10%	20%	40%	80%	100%
Day 0	<0.02	0.20	0.37	0.39	0.76	1.62
Day 7	<0.02	0.10	0.25	0.30	0.89	1.24
Day 14	<0.02	0.33	0.36	0.50	0.82	1.29
Day 28	<0.02	0.08	0.10	0.11	0.33	0.29
Day 42	<0.02	0.06	0.08	0.11	0.25	0.40
Day 56	<0.02	0.05	0.10	0.12	0.80	0.81
Average		0.14	0.21	0.26	0.64	0.94
Std. Dev.		0.11	0.13	0.17	0.28	0.53

Table C-2. 2001 dehydroabietic acid effluent concentrations (all values in mg/L).

	0%	10%	20%	40%	80%	100%
Day 0	<0.02	0.60	1.04	1.18	1.91	3.32
Day 7	1.21	1.61	1.81	1.37	3.12	3.27
Day 14	<0.02	0.82	0.90	1.21	2.59	2.77
Day 28	<0.02	0.07	0.10	0.11	0.32	0.36
Day 42	<0.02	0.06	0.08	0.11	0.20	0.38
Day 56	<0.02	0.05	0.10	0.12	0.20	0.17
Average	0.20	0.46	0.67	0.68	1.39	1.71
Std. Dev.		0.47	0.71	0.63	1.32	1.56

Table C-3. 2001 pimaric acid effluent concentrations (all values in mg/L).

	0%	10%	20%	40%	80%	100%
Day 0	<0.02	0.19	0.33	0.37	0.67	1.33
Day 7	<0.02	0.10	0.23	0.26	0.75	1.17
Day 14	<0.02	0.27	0.31	0.44	0.97	1.20
Day 28	<0.02	0.20	0.26	0.28	0.77	0.71
Day 42	<0.02	0.17	0.22	0.30	0.75	1.01
Day 56	<0.02	0.14	0.27	0.34	0.51	0.43
Average		0.18	0.27	0.33	0.74	0.98
Std. Dev.		0.06	0.04	0.07	0.15	0.34

Table C-4. 2002 isopimaric acid effluent concentrations (all values in mg/L).

	0%	10%	20%	40%	80%	100%
Day 0	<0.02	n/a	0.06	0.11	0.14	0.16
Day 7	<0.02	0.02	0.04	0.06	0.10	0.13
Day 14	<0.02	<0.02	0.02	0.06	0.07	0.07
Day 28	<0.02	0.03	0.03	0.12	0.20	0.22
Day 42	<0.02	<0.02	0.02	0.07	0.10	0.08
Day 56	<0.02	<0.02	0.02	0.08	0.10	0.10
Average		0.03	0.03	0.08	0.12	0.13
Std. Dev.		0.01	0.02	0.03	0.05	0.06

Table C-5. 2002 dehyroabietic acid effluent concentrations (all values in mg/L).

	0%	10%	20%	40%	80%	100%
Day 0	<0.02	n/a	0.04	0.07	0.11	0.11
Day 7	<0.02	0.03	0.07	0.12	0.19	0.24
Day 14	<0.02	0.02	0.04	0.12	0.12	0.13
Day 28	<0.02	0.05	0.07	0.25	0.27	0.44
Day 42	<0.02	<0.02	0.02	0.05	0.09	0.06
Day 56	<0.02	<0.02	<0.02	0.07	0.08	0.08
Average		0.03	0.05	0.11	0.14	0.18
Std. Dev.		0.02	0.02	0.07	0.07	0.14

Table C-6. 2002 pimaric acid effluent concentrations (all values in mg/L).

	0%	10%	20%	40%	80%	100%
Day 0	<0.02	n/a	0.03	0.05	0.07	0.09
Day 7	<0.02	0.02	0.05	0.08	0.13	0.16
Day 14	<0.02	0.02	0.03	0.09	0.11	0.11
Day 28	<0.02	0.04	0.06	0.19	0.22	0.33
Day 42	<0.02	0.00	0.05	0.16	0.24	0.19
Day 56	<0.02	0.06	0.06	0.21	0.23	0.24
Average		0.03	0.05	0.13	0.17	0.19
Std. Dev.		0.02	0.01	0.07	0.07	0.09

Table C-7. 2001 phytosterol concentrations in 100% effluent.

	Campesterol	Stigmasterol	β-Sitosterol	Stigmastanol
Day 0	0.08	0.09	1.02	0.13
Day 7	0.09	0.09	1.06	0.13
Day 14	0.14	0.14	1.74	0.21
Day 28	0.04	0.08	1.41	0.18
Day 42	0.06	0.05	0.70	0.11
Day 56	0.05	0.04	0.47	0.09
Average	0.08	0.08	1.07	0.14
Std. Dev.	0.04	0.03	0.46	0.04

Table C-8. Preliminary β-sitosterol degradation study (AR= aerobic system, AN=anaerobic system).

Hour	AR1	AR2	AN1	AN2
0	93.5	94.6	100	100
43	70.5	92	87.7	85.4
116	77.2	77.6	79.7	71.1
211	35.6	50.2	82.4	80
308	27.9	39.8	75.1	75
360	13.9	38.5	84.9	91.4

Hour	ln AR1	ln AR2	ln AN1	ln AN2
0	4.537961	4.549657	4.30517	4.60517
43	4.255613	4.521789	4.473922	4.447346
116	4.346399	4.351567	4.37827	4.264087
211	3.572346	3.916015	4.411585	4.382027
308	3.328627	3.683867	4.318821	4.317488
360	2.631889	3.650658	4.441474	4.515245

	Half-life (hours)	Half-life (days)	R-squared
AR1	141	5.9	0.9201
AR2	248	10.3	0.9705
AN1	1733	72.2	0.4351
AN2	3465	144.4	0.0695

Table C-9. Definitive β-sitosterol aerobic degradation study results.

Sampling time (hours)	rce1	rce2	rcr1	rcr2	fhr1	fhr2	fhe1	fhe2
				Percent of parent (beta-Sitosterol)				
0	91.1	90.2	88.8	92	87.9	92.1	88.1	90.6
19.5	87	84.2	83.5	81.3	80.1	84	80.6	88.1
69	68.8	75.2	76.3	66.6	71.9	71.1	67.2	78.5
164	57.1	51.7	75.9	54.4	68.2	64.5	54.5	66.1
260	56.3	51.1	55.6	46.2	53.2	62.2	56.7	56.2
500	41.2	46	51.6	44	47.6	56	40.2	43.8
717	34.3	35.6	53.8	45.9	43.8	45.5	41.7	41.1
				Natural log of percent parent molecule				
	4.51	4.50	4.49	4.52	4.48	4.52	4.48	4.51
	4.47	4.43	4.42	4.40	4.38	4.43	4.39	4.48
	4.23	4.32	4.33	4.20	4.28	4.26	4.21	4.36
	4.04	3.95	4.33	4.00	4.22	4.17	4.00	4.19
	4.03	3.93	4.02	3.83	3.97	4.13	4.04	4.03
	3.72	3.83	3.94	3.78	3.86	4.03	3.69	3.78
	3.54	3.57	3.99	3.83	3.78	3.82	3.73	3.72
	rce1	**rce2**	**rcr1**	**rcr2**	**fhr1**	**fhr2**	**fhe1**	**fhe2**
half-life (hours)	533	578	990	770	770	866	693	578
half-life (days)	22.2	24.1	41.3	32.1	32.1	36.1	28.9	24.1
r-squared	0.932	0.860	0.779	0.667	0.897	0.891	0.833	0.933

rce = Rice Creek effluent impacted site.
rcr = Rice Creek reference site.
fhe = Fenholloway River effluent impacted site.
fhr = Fenholloway River reference site.

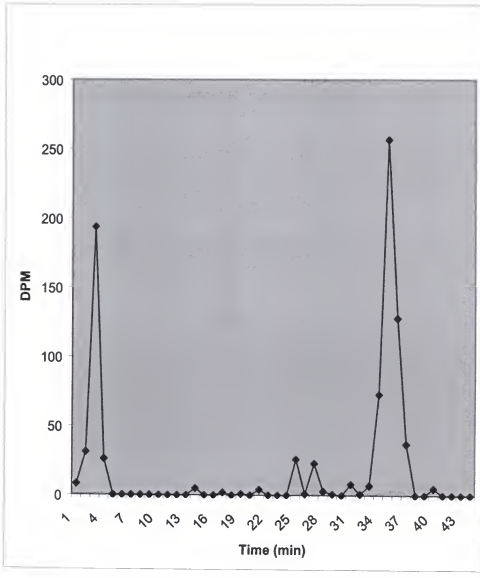


Figure C-1. HPLC histogram for preliminary study (hour 211 aerobic replicate 2).

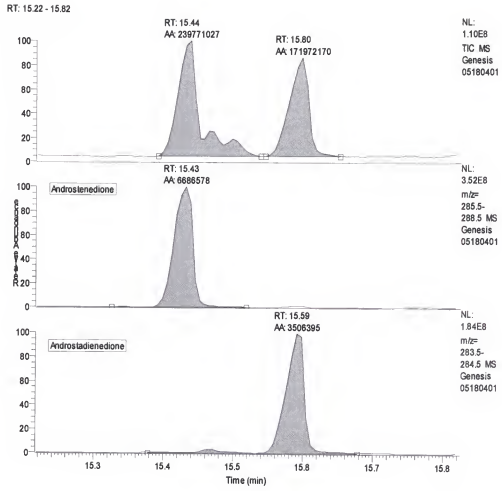
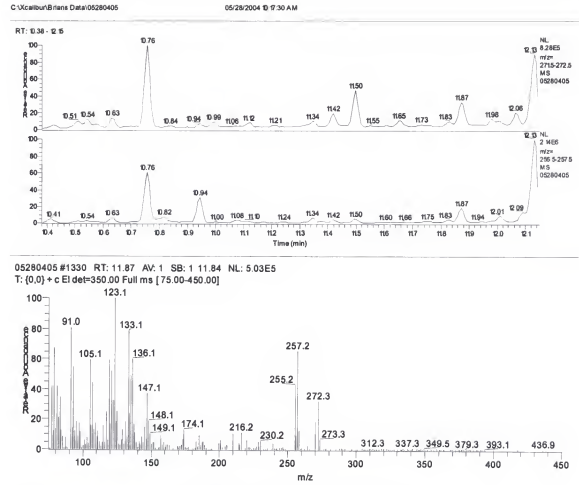


Figure C-2. Androstenedione and androstadienedione standards.



Androsteneone, RT 11.87 (Filename 05280405, BQ Sample 1)

Figure C-3. Androsteneone TIC and mass spectrum.

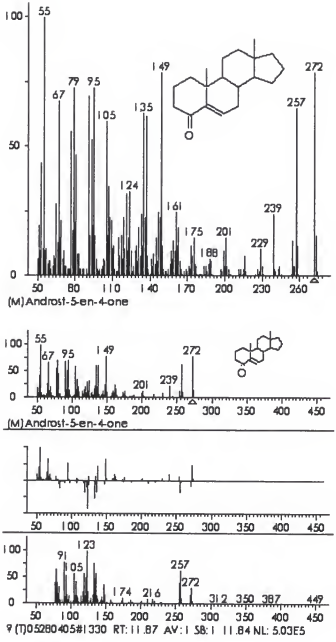


Figure C-4. Androsteneone mass spectrum library match.

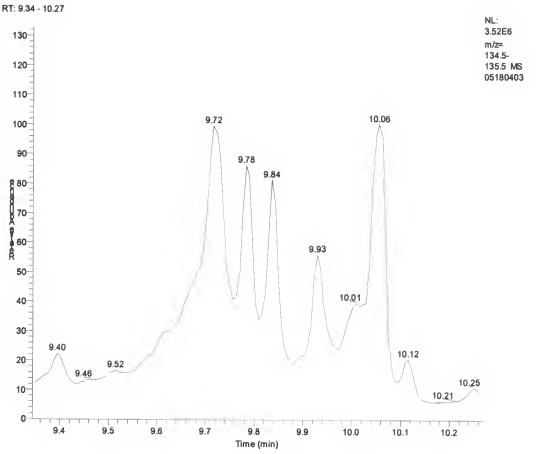


Figure C-5. TIC of nonylphenol.

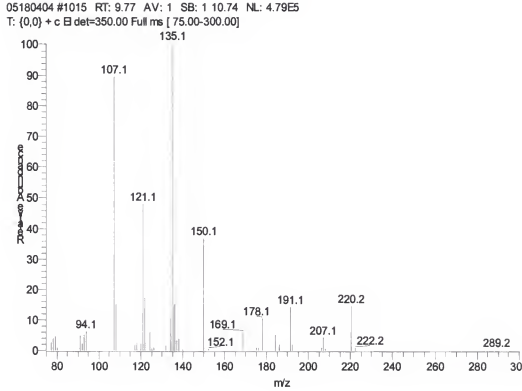


Figure C-6. Mass spectrum of nonylphenol.

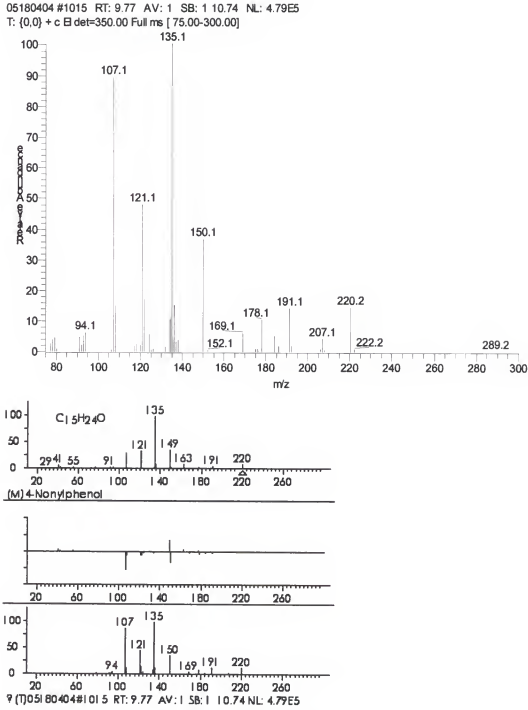


Figure C-7. Nonylphenol mass spectra, EIC, and library match.

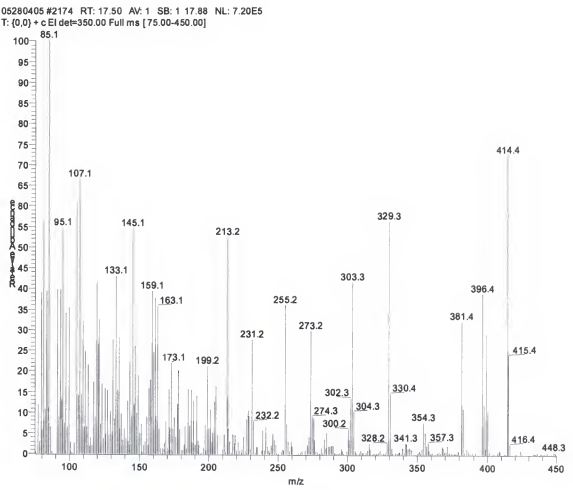


Figure C-8. Mass spectrum of β-sitosterol.

LIST OF REFERENCES

Ambrus, G.; Ilkoy, E.; Jekkel, A.; Horvath, G.; Bocskei, Z. Microbial transformation of β-sitosterol and stigmasterol into 26-oxygenated derivatives. *Steroids.* **1995,** *60,* 621-625.

Arrabal, C.; Cortijo, M. Fatty and resin acids of Spanish *Pinus pinaster* Ait. Subspecies. *JAOCS.* **1994,** *71*(9), 1039-1040.

Awad, A.B.; Sri Hartati, M.; Fink, C.S. Phystosterol feeding induces alteration in testosterone metabolism in rat tissues. *J. Natr. Biochem.* **1998,** *9,* 712-717.

Berryman, D.; Houde, F.; DeBlois, C.; O'Shea, M. Nonylphenolic compounds in drinking and surface waters downstream of treated textile and pulp and paper effluents: a survey and preliminary assessment of their potential effects on public health and aquatic life. *Chemosphere.* **2004,** *56,* 247-255

Bicho, P.A.; Martin, V.; Saddler, J.N. Growth, induction, and substrate specificity of dehydroabietic acid-degrading bacteria isolated from a kraft mill effluent enrichment. *Appl. Environ. Microbiol.* **1995,** *Sept.*, 3245-3250.

Bogdanova, A.Y.; Nikinmaa, M. Dehydroabietic acid, a major effluent component of paper and pulp industry, decreases erythrocyte pH in lamprey (*Lampetra fluviatilis*). *Aquatic Toxicology.* **1998,** *43*, 111-120.

Bortone, S.A.; Cody, R.P. Morphological masculinization in poeciliid females from a paper mill effluent receiving tributary of the St. Johns River, Florida, USA. *Bull. Envrion. Contam. Toxicol.* **1999,** *63,* 150-156.

Brush, T.S.; Farrell, R.L.; Ho, C. Biodegradation of wood extractives from southern yellow pine by *Ophiostoma piliferum. Tappi Journal.* **1994,** *77*(1), 155-159.

Burggraaf, S.; Langdon, A.G.; Wilkins, A.L.; Roper, D.S. Accumulation and depuration of resin acids and fichtelite by the freshwater mussel Hyridella menziesi. *Environ. Toxicol. Chem.* **1996,** *15*(3), 369-375.

Bushnell, P.G.; Nikinmaa, M.; Oikari, A. Metabolic effects of dehydroabietic acid on rainbow trout erythrocytes. *Comp. Biochem. Physiol.* **1985,** *81C*(2), 391-394.

Chow, S.Z.; Shepard, D. High performance liquid chromatographic determination of resin acids in pulp mill effluent. *Tappi Journal.* **1996,** *79*(10), 173-179.

Corin, N.S.; Backlund, P.H.; Kulovaara, M.A.M. Photolysis of the resin acid dehydroabietic acid in water. *Environ. Sci Technol.* **2000,** *34*(11), 2231-2236.

Deardorff, T.L.; Renard, J.J.; Phillips, R.B. An environmental assessment before and after conversion of a bleached kraft mill to elemental chlorine-free bleaching. In *Chlorine and Chlorine Compounds in the Paper Industry*; Turoski, V., ED.; Ann Arbor Press: Chelsea, Michigan, **1998**; pp 143-150.

Denton, T.E.; Howell, W.M.; Allison, J.J.; McCollum, J.; Marks, B. Masculinization of female mosquitofish by exposure to plant sterols and Mycobacterium smegmatis. *Bull. Environ. Contam. Toxicol.* **1985,** *35,* 627-632.

Dethlefs, F.; Stan, H.J. Determination of resin acids in pulp mill EOP bleaching process effluent. *Fresenius J. Anal. Chem.* **1996,** *356*, 403-410.

Dick, T.; Heng Lee, B.; Murugasu-Oei, B. Oxygen depletion induced dormancy in *Mycobacterium smegmatis*. *FEMS Microbiol. Lett.* **1998,** *163,* 159-164.

Dutta, R.K.; Roy, M.; Singh, H.D. Metabolic blocks in the degradation of β-sitosterol by a plasmid-cured strain *Arthrobacter oxydans*. *J. Basic Microbiol.* **1992,** *32,* 167-176.

Elalami, A.; Kreit, J.; Filali-Maltouf, A.; Boudrant, J.; Germain, P. Characterization of a secreted cholesterol oxidase from *Rhodococcus* sp. GK1 (CIP 105 335). *World Journal of Microbiology & Biotechnology.* **1999,** *15,* 579-585.

Farrell, R.L.; Blanchette, R.A.; Brush, T.S.; Hadar, Y.; Iverson, S.; Krisa, K.; Wendler, P.A.; Zimmerman, W. Cartapip™: a biopulping product for control of pitch and resin acid problems in pulp mills. *J. Biotechnology.* **1993,** *30*, 115-122.

Food and Agriculture Organization of the United Nations. *Pulp and paper towards 2010: an executive summary.* Forestry Policy and Planning Division. **1994**: ISBN 92-5-103540-7

Garcia, K.L.; Delfino, J.J.; Powell, D.H. Non-regulated organic compounds in Florida sediments. *Wat. Res.* **1993,** *27*(11), 1601-1613.

Giger, W.; Brunner, P.H.; Schaffner C. 4-nonylphenol in sewage sludge: accumulation of toxic metabolites from nonionic surfactants. *Science.* **1984**, *225*(4662), 623-625.

Giner, J.L.; Dierassi, C. Evidence for sterol side-chain dealkylation in *Chlamydomonas reinhardtii*. *Phytochemistry.* **1992,** *31*(11), 3865-3867.

Guiot, S.R.; Stephenson, R.J.; Frigon, J.C.; Hawari, J.A. Single-stage anaerobic/aerobic biotreatment of resin acid-containing wastewater. *Wat. Sci. Tech.* **1998,** *38*(4-5), 255-262.

Hall, E.R.; Liver, S.F. Interactions of resin acids with aerobic and anaerobic biomass – II. Partitioning on biosolids. *Wat. Res.* **1996,** *30*(3), 672-678.

Howell, W.M.; Denton, T.E. Gonopdial morphogenesis in female mosquitofish, *Gambusia affinis affinis*, masculinized by exposure to degradation products from plant sterols. *Env. Biol. Fish.* **1989,** *24,* 43-51.

Hunsinger, R.N.; Howell, W.M. Treatment of fish with hormones: solubilization and direct administration of steroids into aquaria water using acetone as a carrier solvent. *Bull. Environ. Contam. Toxicol.* **1991,** *47,* 272-277.

James, M. Personal communication **2004**.

Jenkins, R.; Angus, R.A.; McNatt, H.; Howell, W.M.; Kemppainen, J.A.; Kirk, M.; Wilson, E.M. Identification of androstenedione in a river containing paper mill effluent. *Environ. Toxicol. Chem.* **2001,** *20*(6), 1325-1331.

Johnsen, K.; Mattsson, K.; Tana, J.; Struthridge, T.; Hemming, J.; Lehtinen, K.J. Uptake and elimination of resin acids and physiological responses in rainbow trout exposed to total mill effluent from an integrated newsprint mill. *Environ. Toxicol. Chem.* **1995,** *14* (9), 1561-1568.

Johnsen, K.; Tana, J.; Lehtinen, K.J.; Stuthridge, T.; Mattsson, K.; Hemming, J.; Carlberg, G.E. Experimental field exposure of brown trout to river water receiving effluent from an integrated newsprint mill. *Ecotoxicology and Environmental Safety.* **1998,** *40*, 184-193.

Judd, M.C.; Stuthridge, T.R.; Tavendale, M.H.; McFarlane, P.N.; Mackie, K.L.; Buckland, S.J.; Randall, C.J.; Hickey, C.W.; Roper, D.S.; Anderson, S.M.; Steward, D. Bleached kraft pulp mill sourced organic chemicals in sediments from New Zealand rivers. Part 1: Waikato River. *Chemosphere*. **1995,** *30*(9), 1751-1765.

Kendall, R.J.; Brouwer, A.; Giesy, J.P. A risk-based field and laboratory approach to assess endocrine disruption in wildlife. In *Principles and Processes for Evaluating Endocrine Disruption in Wildlife*; Kendall, R., Dickerson, R., Giesy, J., Suk, W., Eds; SETAC: South Carolina, **1996,** pp 1-11.

Kleinow, K.M.; Hummelke, G.C.; Zhang, Y.; Uppu, P.; Baillif, C. Inhibition of P-glycoprotein transport: a mechanism for endocrine disruption in the channel catfish? *Marine Environmental Research.* **2004,** *58,* 205-208.

Kiparissis, Y.; Hughes, R.; Metcalfe, C. Identification of the isoflavonoid genistein in bleached Kraft mill effluent. *Environ. Sci. Technol.* **2001,** *35,* 2423-2427.

Koistinen, J.; Lehtonen, M.; Tukia, K.; Soimasuo, M; Lahtipera, M.; Oikari, A. Identification of lipophilic pollutants discharged from a Finnish pulp and paper mill. *Chemosphere.* **1998,** *37*(2), 219-235.

Krotzer, M.J. The effects of induced masculinization on reproductive and aggressive behaviors of the female mosquitofish, *Gambusia affinis affinis*. *Env. Biol. Fish.* **1990,** *29,* 127-134.

Lamb, D.C.; Kelly, D.E.; Manning, N.J.; Kelly, S.L. A sterol biosynthetic pathway in *Mycobacterium*. *FEBS Letters.* **1998,** *437,* 142-144.

Lee, B.L.; Koh, D.; Ong, H.Y.; Ong, C.N. High-performance liquid chromatographic determination of dehydroabietic and abietic acids in traditional chinese medications. *J. Chromatogr.* **1997,** *763*, 221-226.

Lee, H.B.; Peart, T.E. Supercritical carbon dioxide extraction of resin and fatty acids from sediments at pulp mill sites. *J. Chromatogr.* **1992,** *594*, 309-315.

Lehtinen, K.J.; Mattson, K.; Tana, J.; Engstrom, C.; Lerche, O.; Hemming, J. Effects of wood-related sterols on the reproduction, egg survival, and offspring of brown trout (*Salmo trutta lacustris* L.). *Ecotoxicol. Environ. Saf.* **1999,** *42,* 40-49.

Leppanen, H.; Oikari, A. Occurrence of retene and resin acids in sediments and fish bile from a lake receiving pulp and paper mill effluents. *Environ. Toxicol. Chem.* **1999,** *18* (7), 1498-1505.

Lucas, A.N.; Brogan, L.R.; Nation, R.L.; Milne, R.W.; Evans, A.M.; Shackleford, D.M. The effects of the phytoestrogenic isoflavone genistein on the hepatic disposition of performed and hepatically generated gemifibrozil 1-*O*-acyl glucuronide in the isolated perfused rat liver. *J. Pharm. Pharmacol.* **2003,** *55,* 1433-1439.

Mahato, S.B.; Garai, S. Advances in microbial steroid biotransformation. *Steroids.* **1997,** *62,* 332-345.

Marsheck, W.J.; Kraychy, S.; Muir, R.D. Microbial degradation of sterols. *Applied Microbiology.* **1972,** *22*(1), 72-77.

Martin, V.J.J.; Yu, Z.; Mohn, W.W. Recent advances in understanding resin acid biodegradation: microbial diversity and metabolism. *Arch. Microbiol.* **1999,** *172*, 131-138.

Mattsoff, L.; Oikari, A. Acute hyperbilirubinaemia in rainbow trout (*Salmo gairdneri*) caused by resin acids. *Comp. Biochem. Physiol.* **1987,** *88C*(2), 263-268.

McLeay, D.J.; Walden, C.C.; Munro, J.R. Influence of dilution water on the toxicity of kraft pulp and paper mill effluent, including mechanisms of effect. *Wat. Res.* **1979,** 13, 151-158.

Mesia-Vela, S.; Kauffman, F.C. Inhibition of rat liver sulfotransferases SULT1A1 and SULT2A1 and glucuronosyltransferase by dietary flavonoids. *Xenobiotica.* **2003,** *33*(12), 1211-1220.

Miettinen, V.; Lonn, B.E.; Oikari, A. Effects of biological treatment on the toxicity for fish of combined debarking and kraft pulp bleaching effluent. *Paperi ja Puu – Papper o. Tra.* **1982,** *4*, 251-254.

Morales, A.; Birkholz, D.A.; Hrudey, S.E. Analysis of pulp mill effluent contaminants in water, sediment, and fish bile – fatty and resin acids. *Water Environmental Research.* **1992,** *64*(5), 660-668.

Morgan, C.A.; Wyndham, R.C. Isolation and characterization of resin acid degrading bacteria found in effluent from a bleached kraft pulp mill. *Can. J. Microbiol.* **1996,** *42*, 423-430.

Nakari, T.; Erkomaa, K. Effects of phytosterols on zebrafish reproduction in multigeneration test. *Environmental Pollution.* **2003,** *123,* 267-273.

National Council of the Paper Industry for Air and Stream Improvement, Inc. NCASI. *Procedures for the analysis of resin and fatty acids in pulp mill effluents.* **1986**; Technical Bulletin No. 501. Research Triangle Park, NC.

National Council of the Paper Industry for Air and Stream Improvement, Inc. NCASI. *Resin and fatty acids by extraction/ethylation GC/FID and GC/MS analysis.* **1997**; Method RA/FA-85.02. West Coast Regional Center, Corvallis, OR.

Nieminen, P.; Mustonen, A.M.; Lindstrom-Seppa, P.; Asikainen, J.; Mussalo-Rauhamaa, H.; Kukkonen, J.V.K. Phytosterols act as endocrine and metabolic disruptors in the European polecat (*Mustela putorius*). *Toxicol. Appl. Pharmacol.* **2002,** *178,* 22-28.

Niimi, A.J.; Lee, H.B. Free and conjugated concentrations of nine resin acids in rainbow trout (*Oncorhynchus mykiss*) following waterborne exposure. *Environ. Toxicol. Chem.* **1992,** *11*, 1403-1407.

Nikinmaa, M.; Oikari, A.O.J. Physiological changes in trout (*Salmo gairdneri*) during a short-term exposure to resin acids and during recovery. *Toxicology Letters.* **1982,** *14*, 103-110.

Noggle, J.J.; Smith J.T.; Ruessler, D.S.; Quinn, B.P.; Holm, S.E.; Sepulveda, M.S.; Gross, T.S. Paper mill process modifications reduce biological effects on largemouth bass and Eastern *Gambusia*. *In Pulp & Paper Mill Effluent Environmental Fate & Effects*; Borton, D.L.; Hall, T.J.; Fisher, R.P.; Thomas J.F. ED.; DEStech Publications, Inc.: Lancaster, PA, **2004**; pp 14-24.

Oikari, A.O.J. Metabolites of xenobiotics in the bile of fish in waterways polluted by pulpmill effluents. *Bull. Environ. Contam. Toxicol.* **1986,** *36*, 429-436.

Oikari, A.; Anas, E.; Kruzynski, G.; Holmbom, B. Free and conjugated resin acids in the bile of rainbow trout, *Salmo gairdneri*. *Bull. Environ. Contam. Toxicol.* **1984,** *33*, 233-240.

Oikari, A.; Holmbom, B.; Bister, H. Uptake of resin acids into tissues of trout (*Salmo gairdneri* Richardson). *Ann. Zool. Fennici.* **1982a,** *19*, 61-64.

Oikari, A.; Kunnamo-Ojala, T. Tracing of xenobiotic contamination in water with the aid of fish bile metabolites: A field study with caged rainbow trout (*Salmo gairdneri*). *Aquatic Toxicology.* **1987,** *9*, 327-341.

Oikari, A.; Lindstrom-Seppa, P.; Kukkonen, J. Subchronic metabolic effects and toxicity of a simulated pulp mill effluent on juvenile lake trout, *Salmo trutta* m. *lacustris*. *Ecotoxicology and Environmental Safety.* **1988,** *16*, 202-218.

Oikari, A.; Lonn, B.E.; Castren, M.; Nakari, T.; Snickars-Nikinmaa, B.; Bister, H.; Virtanen, E. Toxicological effects of dehydroabietic acid (DHAA) on the trout, *Salmo gairdneri* Richardson, in fresh water. *Water Res.* **1983,** *17*, 81-89.

Oikari, A.O.J.; Nakari, T. Kraft pulp mill effluent components cause liver dysfunction in trout. *Bull. Environm. Contam. Toxicol.* **1982b,** *28*, 266-270.

Orlando, E.F.; Davis, W.P.; Guillette, L.J. Aromatase activity in the ovary and brain of theeastern mostquitofish (*Gambusia holbrooki*) exposed to paper mill effluent. *Environ. Health Perspect.* **2002,** *110*(3), 429-433.

Owens, J.W.; Swanson, S.M.; Birkholz, D.A. Environmental monitoring of bleached kraft pulp mill chlorophenolic compounds in a northern Canadian river system. *Chemosphere.* **1994a,** *29*(1), 89-109.

Owens, J.W.; Swanson, S.M.; Birkholz, D.A. Bioaccumulation of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, 2,3,7,8-tetrachlorodibenzofuran and extractable organic chlorine at a bleached-kraft mill site in a northern Canadian river system. *Environ. Toxicol. Chem.* **1994b,** *13*(2), 343-354.

Patoine, A.; Manuel, M.F.; Hawari, J.A.; Guiot, S.R. Toxicity reduction and removal of dehydroabietic and abietic acids in a continuous anaerobic reactor. *Wat. Res.* **1997,** *31*(4), 825-831.

Peterman, P.H.; Delfino, J.J.; Dube, D.J.; Gibson, T.A.; Priznar, F.J. Chloro-organic compounds in the lower Fox River, Wisconsin. In *Hydrocarbons and Halogenated Hydrocarbons in the Aquatic Environment*: Afghan, B.K., Mackay, D., Eds.; Plenum Publishing Corporation, **1980,** 149-155.

Quinn, B.P.; Booth, M.M.; Delfino, J.J.; Holm, S.E.; Gross, TS. Selected resin acids in effluent and receiving waters derived from a bleached and unbleached kraft pulp and paper mill. *Environ. Toxicol. Chem.* **2003,** *22*(1), 214-218.

Richardson, D.E.; Bremner, J.B.; O'Grady, B.V. Quantitative analysis of total resin acids by high-performance liquid chromatography of their coumarin ester derivatives. *J. Chromatogr.* **1992,** *595*, 155-162.

Richardson, D.E.; O'Grady, B.V.; Bremner, J.B. Analysis of dehydroabietic acid in paper industry effluent by high-performance liquid chromatography. *J. Chromatogr.* **1983,** *268*, 341-346.

Rogers, I.H. Isolation and chemical identification of toxic components of kraft mill wastes. *Pulp & Paper Magazine of Canada*. **1973,** *74*(9), 1-6.

Sepulveda, M.S. Effects of paper mill effluents on the health and reproductive success of largemouth bass (*Micropterus salmoides*): Field and laboratory studies. Ph.D Thesis. University of Florida, **2000**.

Sepulveda, M.S.; Quinn, B.P.; Denslow, N.D.; Holm, S.E.; Gross, T.S. Effects of pulp and paper mill effluents on reproductive success of largemouth bass. *Environ. Toxicol. Chem.* **2003,** *22,* 205-213.

Soderstrom, M.; Wachtmeister, C.A.; Forlin, L. Analysis of chlorophenolics from bleach kraft mill effluents (BKME) in bile of perch (Perca fluviatilis) from the Baltic Sea and development of an analytical procedure also measuring chlorocatechols. *Chemosphere*. **1994,** *28*(9), 1701-1719.

Suckling, I.D.; Gallagher, S.S.; Ede, R.M. A new method for softwood extractives analysis using high performance liquid chromatography. *Holzforschung*. **1990,** *44*(5), 339-345.

Suntio, L.R.; Shiu, W.Y.; Mackay, D. A review of the nature and properties of chemicals present in pulp mill effluents. *Chemosphere*. **1988,** *17*(7), 1249-1290.

Tavendale, M.H.; Hannus, I.M.; Wilkins, A.L.; Langdon, A.G.; Mackie, K.L.; McFarlane, P.N. Bile analyses of goldfish (Crassius auratus) resident in a New

Zealand hydrolake receiving a bleached kraft mill discharge. *Chemosphere*. **1996,** *33*(11), 2273-2289.

Tavendale, M.H.; McFarlane, P.N.; Mackie, K.L.; Wilkins, A.L.; Langdon, A.G. The fate of resin acids-1. The biotransformation and degradation of deuterium labelled dehydroabietic acid in anaerobic sediments. *Chemosphere*. **1997a**, *35*(10), 2137-2151.

Tavendale, M.H.; McFarlane, P.N.; Mackie, K.L.; Wilkins, A.L.; Langdon, A.G. The fate of resin acids-2. The fate of resin acids and resin acid derived neutral compounds in anaerobic sediments. *Chemosphere*. **1997b**, *35*(10), 2153-2166.

Tavendale, M.H.; Wilkins, A.L.; Langdon, A.G.; Mackie, K.L.; Stuthridge, T.R.; McFarlane, P.N. Analytical methodology for the determination of freely available bleached kraft mill effluent-derived organic constituents in recipient sediments. *Environ. Sci. Technol*. **1995,** *29*, 1407-1414.

Tremblay, L.; Van Der Kraak, G. Comparison between the effects of the phytosterol ß-sitosterol and pulp and paper mill effluents on sexually immature rainbow trout. *Environ. Toxicol. Chem.* **1999,** *18*(2), 329-336.

US EPA. *Profile of the pulp and paper industry*. **1995**; U.S. Environmental Protection Agency. Office of Compliance. EPA 310-R-95-015, Washington D.C.

US EPA. *Pulp and paper NESHAP microform: a plain English description*. **1998**; U.S. Environmental Protection Agency. Office of Air Quality Planning and Standards. Washington D.C.

Van Der Geize, R.; Hessels, G.I.; Van Gerwen, R.; Vrijbloed, J.W.; Van Der Meijden, P.; Dijkhuizen, L. Targeted disruption of the *ks*D gene encoding a 3-ketosteroid Δ^1-dehydrogenase isoenzyme of *Rhodococcus erythropolis* strain SQ1. *Appl. Environ. Microbiol.* **2000,** *66*(5), 2029-2036.

Voss, R.H.; Rappsomatiotis, A. An improved solvent-extraction based procedure for the gass chromatographic analysis of resin and fatty acids in pulp mill effluents. *J. Chromatogr*. **1985,** *346*, 205-214.

Weser, U.; Kaup, Y.; Etspuler, H.; Koller, J.; Baumer, U. Embalming in the old kingdom of pharaonic Egypt. *Analytical Chemistry*. **1998,** *August 1*, 511-516.

White R.; Jobling, S.; Hoare, S.A.; Sumpter, J.P.; Parker, M.G. Environmentally persistent alkylphenolic compounds are estrogenic. *Endocrinology*. **1994**, *135*(1), 175-182.

Wilson, A.E.J.; Moore, E.R.B.; Mohn, W.W. Isolation and characterization of isopimaric acid-degrading bacteria from a sequencing batch reactor. *Appl. Environ. Microbiol.* **1996,** 3146-3151.

Zanella, E. Effect of pH on acute toxicity of dehydroabietic acid and chlorinated dehyroabietic acid to fish and *Daphnia*. *Bull. Environm. Contam. Toxicol.* **1983,** *30*, 133-140.

Zender, J.A.; Stuthridge, T.R.; Langdon, A.G.; Wilkins, A.L.; Mackie, K.L.; McFarlane, P.N. Removal and transformation of resin acids during secondary treatment at a New Zealand bleached kraft pulp and paper mill. *Wat. Sci. Tech.* **1994,** *29*(5-6), 105-121.

Zhang, Y.; Bicho, P.A.; Breuil, C.; Saddler, J.N.; Liss, S.N. Resin acid degradation by bacterial strains grown on CTMP effluent. *Wat. Sci. Tech.* **1997,** *35*(2-3), 33-39.

Zheng, J.; Nicholson, R.A. Action of resin acids in nerve ending fractions isolated from fish central nervous system. *Environ. Toxicol. Chem.* **1998,** *17*, 185

BIOGRAPHICAL SKETCH

I was born in Salisbury, Missouri on May 5, 1967 to George and Virginia Quinn as their youngest child and only son. I grew up in rural Salisbury, located in North-central Missouri, playing sports and enjoying the outdoors. I attended Salisbury High School from 1981-1985 and became interested in science, which led me to major in biology at the University of Missouri-Columbia. I graduated from MU in 1990 and obtained a job with a local contract laboratory, ABC Laboratories, where I was an environmental fate chemist. From Columbia, I moved to Jupiter, Florida in 1991 to pursue a career in environmental chemistry at Toxikon Environmental Sciences. After three years in South Florida, I moved to Gainesville, Florida to work for and attend the University of Florida. I graduated with my masters degree in environmental engineering sciences in August 2000 and have spent the last 4 years working on my doctoral degree. I married Nicola Kernaghan in 1997 and we reside in rural Alachua County near the town of Alachua. My hobbies include gardening, native plant botany, music, food, fishing, and rugby.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Joseph J. Delfino, Chair
Professor of Environmental Engineering Sciences

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Timothy S. Gross, Cochair
Associate Scientist of Veterinary Medicine

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Paul A. Chadik
Associate Professor of Environmental Engineering Sciences

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

David H. Powell
Scientist of Chemistry

This dissertation was submitted to the Graduate Faculty of the College of Engineering and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August 2004

Pramod P. Khargonekar
Dean, College of Engineering

Kenneth J. Gerhardt
Interim Dean, Graduate School